JUN 25 1925

VOL. VI. No. 3] [JUNE, 1925.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

Editors:

C. L. EVANS
P. FILDES
W. E. GYE
E. H. KETTLE
J. McINTOSH
H. MacLEAN
J. A. MURRAY
J. B. ORR
W. J. TULLOCH
C. M. WILSON

With the Support of:

F. W. ANDREWES (London)
A. E. BOYCOTT (London)
W. BULLOCH (London)
S. L. CUMMINS (Cardiff)
H. H. DALE (London)
H. R. DEAN (Cambridge)
A. M. DRENNAN (Otago, N.Z.)
G. DREYER (Oxford)
J. W. H. EYRE (London)
LEONARD FINDLAY (Glasgow)
DUNCAN GRAHAM (Toronto)
R. T. HEWLETT (London)
F. G. HOPKINS (Cambridge)
C. H. KELLAWAY (Melbourne)
J. C. G. LEDINGHAM (London)
W. B. LEISHMAN (London)
T. J. MACKIE (Edinburgh)
H. OERTEL (Montreal)
LEONARD ROGERS (London)
C. S. SHERRINGTON (Oxford)
A. E. WRIGHT (London)

LONDON: H. K. LEWIS & CO. LTD., 136, GOWER STREET, W.C.1.
EDITORIAL AND PUBLISHING OFFICE: 28, GOWER PLACE, W.C.1.
CANADA: THE MACMILLAN COMPANY OF CANADA, LTD., BOND STREET, TORONTO.

Annual Subscription, January to December, post free, Two Pounds net.
Single Numbers, Seven and Sixpence net.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY.

EDITORIAL NOTICE TO CONTRIBUTORS.

Scope of Papers.—This Journal was founded for the publication of original communications describing the technique and results of experimental researches into the causation, diagnosis and cure of disease in man. Papers upon bacteriological, biochemical, physiological, serological and other subjects are therefore acceptable when bearing directly upon these objects.

Forwarding Papers.—Papers should be addressed to—

THE EDITORS,

British Journal of Experimental Pathology,

28, GOWER PLACE,

LONDON, W.C. 1,

or to any of the Editors personally.

Date of Publication.—The Journal is published bi-monthly, in February, April, June, August, October, and December.

Priority of Publication.—Papers accepted for publication will be printed as far as possible in the order of their receipt by the Editors, but those sent from overseas will receive priority in publication according to the date of posting instead of the date of receipt.

Conditions of Publication.—Contributors should prepare their manuscripts with the greatest attention to detail. The fact of submitting a paper for publication will be taken by the Editors as an admission by the Author of his liability to pay for all unreasonable alterations made by him in proofs, and for mistakes due to lack of clearness in the preparation of the manuscript.

References.—Authors are requested to prepare their references in strict accordance with the method adopted in current issues of this Journal.

Proofs.—To avoid delay, proofs will not be submitted to Authors overseas unless they express a desire to see them, or unless the Editors think it necessary. Overseas Authors are therefore particularly reminded of the importance of preparing their manuscripts in such a way that error need not arise.

Reprints.—25 reprints will be supplied free to authors. Additional copies must be ordered from the Publishers *before the issue is printed.*

IMPORTANT NOTICE.

To ensure rapid publication, contributors should be concise in the presentation of their results. Reviews of the literature and theoretical discussions should be reduced to the minimum.

Made and printed in Great Britain.

THE BRITISH JOURNAL OF EXPERIMENTAL PATHOLOGY

VOL. VI JUNE, 1925 No. 3

TETANUS.—II. AGGLUTINATION AND TOXICITY OF *B. TETANI.*

PAUL FILDES.

From the Bacteriological Department, London Hospital.

Received for publication March 1st, 1925.

IN a previous paper (Fildes, 1925) it was stated that a large number of strains morphologically and culturally resembling tetanus bacilli but without demonstrable toxicity were identified as *B. tetani* from the results of agglutination tests.

In the present paper the details are presented.

Agglutination was employed by Tulloch (1917, 1917–19[1], 1917–19[2], 1919) as a method of detecting *B. tetani* in cultures made from wounds during the war. The advantage of the method was that it was applicable to impure cultures "enriched" by the use of a selective medium. Three strains isolated by Miss Robertson with Barber's single-cell method were used as "type" cultures for preparing agglutinating sera in rabbits.

Employing these for examining the toxic laboratory strains that were then available and also the impure cultures which he had at that time obtained from wounds, Tulloch found that all agglutinated with one or other of the three sera.

Later a fourth type (IV) was encountered, but was observed only five times.

I am informed by Dr. Tulloch that a further strain (type V) was received by him from the late Major Graeme Gibson in France after completion of his report to the Tetanus Committee. This did not agglutinate with any of the four type sera; it was, however, markedly toxic, and rapidly agglutinated with a serum prepared against itself.

Agglutination was also used by Bauer (1924) to test the types of the strains obtained by Tenbroeck and Bauer (1922) from the fæces of Chinese. He found no example of type II or of type IV, but the commonest was the type V not found by Tulloch.

The types with which I have worked have been types I, II and III isolated by Miss Robertson and acquired from the National Collection of Type Cultures (N.C.T.C. Nos. 279, 540 and 539 respectively) and types IV and V received from Dr. Tulloch and purified by myself.

In order to test the value of this method for identification, all bacilli which had spherical or almost spherical terminal spores were isolated, and without reference to their cultural characters were tested against five monotypical sera corresponding to the five types and one polyvalent serum made by immunizing rabbits with a mixture of the five types.

Technique.—The production of agglutinins for tetanus bacilli in rabbits and the agglutination of the bacilli is so simple that precise details are unnecessary.

The inoculum was a suspension from peptic blood-agar plates (4 days' growth), washed off with saline, centrifuged and made up with saline to standard opacity.* This was heated to 60° C. for 1½ hours and then carbolized (0·5 per cent.). Rabbits were inoculated intravenously at four-day intervals with 1, 2 and 3 c.c. of this suspension. Titres of 1/1600 were obtained—sometimes higher, sometimes lower. At first the same suspension was used for agglutination, diluted 1/3 with saline, but it was found that spontaneous agglutination always occurred with type III strains.† This was found to be due to the slight washing with saline. The bacilli should, therefore, for agglutination be washed off with saline and made up to the correct opacity without centrifuging. A more convenient and apparently excellent technique, however, is to use a liquid culture undiluted, unheated and not carbolized. The agglutination of tetanus bacilli is always very massive and takes place rapidly at 56° C. One hour was taken as the limit of time.

In Tulloch's publications on types I, II, III and IV there is shown to be a marked specificity to type—that is to say, there is no cross-agglutination of other types by a serum of one type in a dilution of 1/100. This I have been unable altogether to confirm. I have found that the type III serum agglutinates also type II, and further, that the type V serum, with which Tulloch did not work, agglutinates type IV as shown in Table I.‡

TABLE I.—*Cross-agglutination of Type Strains.*

The figure gives the dilution of serum causing complete agglutination in a broth culture in one hour at 56° C.; lowest dilution 1/100, highest 1/1600.

		Type suspensions.				
		I.	II.	III.	IV.	V.
Sera:	I	1600	0	0	0	0
	II	0	800	0	0	0
	III	0	200	800	0	0
	IV	0	0	0	400	0
	V	0	0	0	400	1600
	0	0	0	0	0	0

The antigenic structure of the five type-strains may, however, be shown to be distinct by absorption of agglutinin tests.

* The standard opacity was that of a mixture of equal parts of N/100 $BaCl_2$ and N/100 H_2SO_4.

† This fact was also noted by Bauer (1924), who could not, however, avoid it.

‡ In reference to Tulloch's type strains Bauer (1924) says, "The differentiation of the types is quite striking as the other organisms were not, as a rule, clumped in dilution as low as 1/100."

Technique.—In order to extract fully the agglutinin from the serum it was found necessary to carry out the process twice. A polyvalent serum was used rather than five monotypical sera for the reasons given in another connection (Fildes, 1920). The standard suspension of each bacillus was centrifuged, the supernatant discarded, and the volume made up again with polyvalent serum 1/50. After standing for one hour at room temperature and for 15 minutes at 56° C. the tubes were centrifuged, and the supernatant transferred to another deposit of bacilli. After the lapse of the same periods of time the final supernatant was tested as absorbed serum, in dilutions, which, when mixed with an equal volume of bacilli, gave a range from 1/100 to 1/1600.

In this manner it was shown that each type-strain removed its own agglutinin entirely from the polyvalent serum, while it did not reduce the titre of the serum for the other type-strains in the slightest degree.

During the course of this work six strains were met with which agglutinated with types II and III sera (TH 8, TH 9, TH 10, TH 11, TH 16 and TS 31). These were shown by absorption tests to belong to type II. No specimens were encountered which agglutinated with type II serum alone. Four were found which agglutinated with types IV and V sera, but none with type IV alone. In Table II is shown the result of the absorption tests upon these four strains.

TABLE II.—*Absorption Tests on Strains Agglutinating with Types IV and V Sera.*

Type suspensions.	Polyvalent serum absorbed with— 0	TS 14.	TS 20.	TS 22.	TS 23.
I	+ + + + +	+ + + + +	+ + + + +	+ + + + +	+ + + + +
II	+ + + ⊥ 0	+ + + ⊥ 0	+ + + ⊥ 0	+ + + ⊥ 0	+ + + ⊥ 0
III	+ + + + ⊥	+ + + + ⊥	+ + + + ⊥	+ + + + ⊥	+ + + + ⊥
IV	+ + + ⊥ 0	0 0 0 0 0	0 0 0 0 0	+ + + ⊥ 0	0 0 0 0 0
V	+ + + + +	+ + ⊥ 0 0	+ + + + 0	+ + 0 0 0	+ 0 0 0 0

+ = complete agglutination after 1 hour at 56° C. = ⊥ incomplete agglutination visible to the naked eye.

It would appear from Table II that TS 22 is a pure type V strain, while the other three have a varying degree of antigenic relationship to types IV and V. Thus no instance of pure type IV has been met with.

The polyvalent serum was made in the hope of obtaining agglutination with strains which would not agglutinate with monotypical sera, but this hope was not realized. Rabbits were immunized with a mixture containing three varieties of types I, II, III and V and one variety of type IV. The serum obtained agglutinated all strains which agglutinated with monotypical sera, but did not agglutinate any strain which failed to react with monotypical sera.

Table III indicates the results obtained when testing by agglutination all strains of bacilli which more or less resembled tetanus bacilli in morphology. Strains agglutinating with more than one monovalent serum were checked by absorption tests.

TABLE III.—*Types and Toxicity of Bacilli found in possibly Tetanic Material.*

	Types indicated by agglutination or absorption tests: I T	I N	II T	II N	III T	III N	IV T	IV N	IV-V T	IV-V N	V T	V N		No agglutination with type or polyvalent sera. T	N
Tulloch's strains	1	0	1	0	1	0	0	1*	—		1	0			—
Cases of tetanus	2	1*	0	0	2	0	0	0	0	0	1	0	5 toxic, 1 non-toxic.	0	0
Horse fæces	6	2	5	0	6	1	0	0	0	0	8	6	25 toxic, 9 non-toxic.	0	9
Human fæces	0	0	0	0	0	0	0	0	0	0	2	0	2 toxic.	0	4
Soil	0	2	1	0	4	1	0	0	0	3	8	14	13 toxic, 20 non-toxic.	0	1
	8		6		12		0		0		19		45		
		5		0		2		0		3		20	30		
	13		6		14		0		3		39		75		

T = toxic; N = non-toxic. * toxic when first isolated.

It will be seen that 45 of these strains of bacilli were toxic, and by this character are proved to be tetanus bacilli. All of these strains agglutinated readily with polyvalent serum, or with the monovalent sera. Thus it would seem that sera made from the 5 types, either mono- or polyvalent, are adequate for the diagnosis of all undoubted tetanus bacilli so far met with.

The position of those strains, 30 in number, which agglutinate with tetanus serum, but which are described as non-toxic, is less certain. In this paper they are looked upon as tetanus bacilli which fail to exhibit the character of toxicity, relying on the precedent of *B. diphtheriæ*. In the case of tetanus this assumption is, perhaps, more justifiable than in diphtheria, because in the former there is clear evidence (p. 97) that toxic strains can become non-toxic, whereas in the latter this is not so certain. The description of the characters of *B. tetani* contained in my former paper (Fildes, 1925) was based upon the behaviour of the whole group of 75 agglutinating organisms, and it was shown that there was no essential difference between the strains which were toxic and the non-toxic.

With regard to the agglutinogenic type of the bacilli encountered it is seen that the commonest was type V. This was also the commonest in Bauer's (1924) series, but Tulloch did not meet with it at all.

Fourteen strains of bacilli are shown in the table as not agglutinating with tetanus sera and as being non-toxic. These are strains which bore a more or less close morphological resemblance to *B. tetani*, and for this reason were studied further. They may be divided roughly into two groups:

(1) Those in which the morphological resemblance to *B. tetani* is close, the effect upon sugars and gelatine the same and the type of film growth somewhat similar. Six strains from horse-dung and one (41 S) from soil may be included in this group, but only in the case of the latter was a mistake in diagnosis actually made. The most valuable distinction is the absence of the filamentous edge to the spreading film. The relationship of these strains to *B. tetani* is not of much practical importance. There is no reason to look upon them as tetanus bacilli belonging to a type not already recognized until a toxic non-agglutinating strain is found among them.

(2) The second group also consists of 7 strains, 4 from human fæces and 3 from horse-dung. These are more unlike tetanus bacilli in morphology and

grow with an entirely different type of surface film. They did not liquefy gelatine, though growth was feeble. One fermented the four test-sugars and one glucose and lactose.

It seems probable that some or all of these non-agglutinating bacilli correspond to the various tetanus-like bacilli of the literature, namely, *B. putrificus*, *B. tetanomorphus*, *B. cochlearius*, etc., but at the same time it must be confessed that the descriptions of some of these bacteria in the literature are hardly adequate to allow any exact identification.

The most practical points which arise out of the study of agglutination as applied to tetanus-like bacilli are: (1) agglutination with a polyvalent serum made from a mixture of strains belonging to the 5 types is a satisfactory criterion of *B. tetani*, and (2) a large number of tetanus bacilli have no demonstrable toxicity.

TOXICITY OF *B. TETANI*.

As indicated in Table III, of 75 strains which agglutinated with tetanus sera 45 were toxic and 30 non-toxic.

Criteria of toxicity.—The toxicity of strains was primarily determined by injecting 1 c.c. of a 7–21-day liquid culture into the muscles on the inner aspect of the right thigh of guinea-pigs. The medium was ordinary broth enriched with the peptic digest of blood.

A control using an immunized animal was considered unnecessary.

Forty strains out of 75 produced tetanus under these conditions.

The effect of the injection of a toxic culture was very constant. The animals sometimes died within 24 hours or were killed in 24 hours in a state of general tetanus. In some cases only local tetanus appeared in 24 hours, but if left these animals were dead next morning. In only two instances did it happen that an animal developed tetanus later than 36 hours after injection (3 days), although survivors were observed for weeks and sometimes months.

The lesions found in the animals *post mortem* were often more severe than accounts in the literature would suggest, this no doubt being due to the largeness of the dose injected. In most cases no conspicuous lesion was found, but in some there was a definite œdema in the groin and surrounding tissues, occasionally slightly blood-stained. No localized lesion was found in the muscles of the right thigh, and it was very uncommon to find any bacilli microscopically in this situation.

Cultures were taken from the heart-blood and liver of all animals which were killed. Tetanus bacilli were never found in the heart-blood. Practically the whole of the left lower lobe of the liver was taken, and in 14 out of 37 animals tetanus bacilli were found in pure culture. Cultures were seldom taken from the right thigh, but were always positive.

These results are in general agreement with such accounts in the literature as are comparable. Tizzoni, Cattani and Baquis (1890) found the spleens of two animals positive out of 6 injected with pure cultures, and 4 out of 6 with impure cultures. Vaillard and Vincent (1891) rarely found tetanus bacilli in the organs after intramuscular injection, and never in the blood. Oettingen and Zumpe (1899) removed the whole heart and spleen from 45 animals injected with impure cultures and found 20 positive mixed with other bacteria.

NON-TOXIC STRAINS.

The large number of non-toxic strains revealed by the guinea-pig test was a matter for surprise, particularly since two strains, TM 1 and TM 5, actually had been isolated from cases of tetanus. It was thought that the guinea-pig might be an unsuitable test animal, and therefore all non-toxic strains were re-tested upon mice on two occasions, once with 1 c.c. of a 14-day peptic-blood broth culture and once with 1 c.c. of a 14-day coagulated serum culture. The inoculation was made subcutaneously at the base of the tail. In these tests the following strains produced general tetanus in 24 hours: TM 5, TN 1 and TN 2; TH 23 produced fatal tetanus on the 6th day, while TH 24 produced local tetanus after 6 days, which did not progress for a further 6 days, when the animal was killed.

It seems likely that this disclosure of a few additional toxic strains is due not to any special susceptibility of the mouse, but merely to the relatively greater dose.

In view of the possibility that the demonstrated lack of toxicity to guinea-pigs and mice might be due to some error in the composition of the medium, a number of strains were cultivated in different media and for different times from 3 to 40 days. Several preparations of digested and undigested blood or digested and undigested serum were tried, but in no case was evidence of toxin production obtained. In a few instances as much as 3 c.c. of a culture was inoculated into a guinea-pig.

Tulloch (1919) has suggested a distinction between the toxigenic function of tetanus bacilli *in vitro* and their power of producing tetanus in susceptible animals. Experiments were, therefore, carried out to test whether these non-toxic strains could be shown to be pathogenic when injected with "tissue-debilitants." The debilitant chiefly used was calcium chloride (Bullock and Cramer, 1919) in doses varying between 0·5 c.c. of a 1 per cent. solution and 0·5 c.c. of a 5 per cent., but in a few cases saponine, as advised by Tulloch, was used. These substances were injected with half a slope culture 4 days old or with 1 c.c. of a 7-day liquid culture. No tetanus was, however, produced by 13 non-toxic strains tested under these conditions.

Negative results were also obtained with mixed broth cultures of the test strains and *Staphylococcus aureus*.

A distinction between toxicity and virulence, therefore, could not be demonstrated in these experiments.

The large number of non-toxic strains found in this series was not to be expected from previous accounts in the literature. Isolated examples have, of course, been described, but in the absence of an agglutination test it is impossible to be certain of the relationship of the organisms to *B. tetani*. The bacillus of Tavel (1898) obviously was not tetanus and was not claimed to be. Sanfelice's anaerobe IX (1893) may have been a non-toxic tetanus bacillus, but the four strains of Adamson and Cutler (1917) differ from tetanus bacilli, as the authors state. Semple (1911) claimed to have isolated a non-toxic tetanus bacillus from the fæces of a guinea-pig. Douglas, Fleming and Colebrook (1920) described a bacillus, Tholby I, which differed from tetanus only in the absence of toxicity. Heller (1922) stated that she isolated 6

non-toxic mutants from a single toxic culture. Turning to more extensive observations, Tenbroeck and Bauer (1922) examined the stools of a large number of Chinese for tetanus bacilli, and from 30 which showed drum-sticks in mixed culture they isolated 29 strains toxic to mice. No non-toxic strains were mentioned. Tulloch, also, does not appear to have met with non-toxic strains comparable with those mentioned in this paper.

SPONTANEOUS LOSS OF TOXICITY OF TETANUS BACILLI.

The fact that a few strains were only slightly toxic, as indicated by the late onset of the disease or the large dose necessary, suggests that there may be a gradual change from toxic to non-toxic. The following case, however, supplies direct evidence of this :

A strain (TM 1) isolated *post mortem* by myself in 1921 from a case of human tetanus when tested upon a guinea-pig was shown to be toxic. It was preserved at room temperature in the dark in boiled-blood broth until October, 1923, when 1 c.c. of a 4-day peptic-blood broth culture was injected into a guinea-pig. No effect was produced. Six further tests were made subsequently with inocula of different age and constitution with and without debilitants, but without result. Finally, in January, 1925, 1 c.c. of a heavy 10-day tryptic digest of serum culture was injected into a mouse, also without effect. It is thus clear that a strain which at first was presumably toxic to man and certainly toxic to a guinea-pig may, simply with the lapse of time, become non-toxic to guinea-pigs and be non-toxic to mice.

A similar phenomenon was encountered with type strain IV, though it is not so certain because the original test was made on a rat. In a private communication Dr. Tulloch states that this strain was toxic when isolated, but on 7 different occasions I have found no toxicity for guinea-pigs and twice no toxicity for mice. Since isolation it was preserved as an impure suspension sealed in a capsule.

THE RELATION OF TOXICITY TO ANIMALS TO TOXICITY TO MAN.

It seems very doubtful whether lack of toxicity to animals may safely be taken to imply harmlessness to man. The case of TM 5 is an instance of the necessity for caution. This bacillus was isolated from the wound dressings of a boy aged 6 years, who had received a small laceration of the eye-brow in a street accident. The onset of the disease was 6 days after the injury, the culture was taken on the 7th day (25 . ix . 24), and he died on the 9th day. The strain was type V. On 21 . x . 24, 1 c.c. of a 7-day liquid culture was inoculated into a guinea-pig, and on 19 . xii . 24, 1 c.c. of a 14-day culture. No effect was produced. On 19 . i . 25, 1 c.c. and 3 c.c. of a 10-day tryptic digest of serum culture were inoculated into two guinea-pigs, and on 21 . i . 25, 1 c.c. of the same digest into a mouse. The guinea-pigs remained well, and the mouse was killed while in general tetanus in 24 hours. Thus this strain killed a boy aged 6 years, and a mouse, but not a guinea-pig.

It is obvious that the conditions under which a bacillus produces tetanus in nature are not the same as those under which laboratory tests are carried out.

The decision on the harmlessness to man of supposed non-toxic strains must await the discovery of the mechanism by which infection takes place in man.

CONCLUSIONS.

(1) All of 75 recently isolated strains, corresponding in morphology and cultural reactions with *B. tetani*, agglutinated with sera made from 5 type-strains.

(2) No example identical with Tulloch's type IV was met with.

(3) Agglutination with a polyvalent serum made from the 5 type-strains is a satisfactory criterion of a tetanus bacillus.

(4) 45 of the new strains were toxic to guinea-pigs or mice; 30 had no demonstrable toxic effect upon these animals.

(5) Spontaneous loss of toxicity may occur.

(6) It is unsafe to assume that strains non-toxic to animals are non-toxic to man.

The cultures referred to have been deposited in the National Collection of Type Cultures, as indicated in my previous paper.

This work was carried out under grants from the Medical Research Council.

I have to express my thanks to Dr. Tulloch for providing me with cultures and information.

REFERENCES.

Adamson, R. S., and Cutler, D. W.—(1917) *Lancet*, **1**, 688.
Bauer, J. H.—(1924) *Proc. Soc. Exp. Biol. & Med.*, **21**, 265.
Bullock, W. E., and Cramer, W.—(1919) *VIth Sci. Rep. Imp. Cancer Res. Fund.* London (Taylor & Francis).
Douglas, S. R., Fleming, A., and Colebrook, L.—(1920) *Med. Res. Council; Spec. Rep. Series*, No. 57. London (H.M. Stationery Office).
Fildes, P.—(1920) *Brit. J. Exper. Pathol.*, **1**, 44.—(1925) *Ibid.*, **6**, 62.
Heller, H. H.—(1922) *J. Infect. Dis.*, **30**, 18.
Oettingen, W., and Zumpe, C.—(1899) *Deutsch. Arch. f. klin. Med.*, **64**, 478.
Sanfelice, F.—(1893) *Ztschr. f. Hyg. u. Infectionskr.*, **14**, 339.
Semple, D.—(1911) *Sci. Mem. Officers of the Med. and San. Dept., Govnmt. of India*, No. 43. Calcutta.
Tavel, E.—(1898) *Cntrlbl. f. Bakt.*, **23**, 538.
Tenbroeck, C., and Bauer, J. H.—(1922) *J. Exp. Med.*, **36**, 261.
Tizzoni, G., Cattani, J., and Baquis, E.—(1890) *Beitr. z. path. Anat. u. z. allg. Path.*, **7**, 568.
Tulloch, W. J.—(1917) *J. R. Army Med. Corps*, **29**, 631.—(1917–19[1]) *Proc. R. Soc.*, B, **90**, 145.—(1917–19[2]) *Ibid.*, **90**, 529.—(1919) *J. Hyg.*, **18**, 103.
Vaillard, L., and Vincent, H.—(1891) *Ann. de l'Inst. Pasteur*, **5**, 1.

THE ANTIGENIC PROPERTIES OF LYSOZYME-DISSOLVED VACCINES.

V. DOUGLAS ALLISON, M.D.,
Beit Memorial Research Fellow.

From the Laboratories of the Institute of Pathology and Research, St. Mary's Hospital, London.

Received for publication March 20th, 1925.

In testing the potency of an anti-bacterial serum or the degree of immunity conferred after a series of injections with a bacterial vaccine there is no generally accepted method, but we have at our disposal the titration of one or more of the several antibodies produced. It has been assumed by various authorities that when a bacterial antigen is injected into the body it comes into intimate contact with various agencies in the body-tissues and fluids, which, by causing a gradual solution of the organisms, stimulate the production of the various antibodies; in other words, the antigen only becomes effective after it has been dissolved by the various tissue-cells and fluids. Sanarelli, Jobling and Strouse, Rosenow, Douglas, Dreyer and other workers have carried out experiments to test the antigenic properties of bacterial vaccines, which had been partially or completely digested and dissolved by various agents; most of these experiments were conducted with a view to finding out whether digestion or solution of the organisms had any effect on their toxicity or their power to stimulate the production of antitoxins, and the organisms used were therefore highly pathogenic, *e. g. V. choleræ*, *B. typhosus*, pneumococcus, etc. Douglas (1921), however, studied the general antigenic properties in addition to the toxicity, in respect of organisms extracted with acetone and digested by trypsin. This work will be referred to later.

The recent experiments of Fleming (1922), and Fleming and Allison (1923[1, 2], 1924), have demonstrated the presence, in practically all the tissues and secretions of the human and animal body, of a substance—lysozyme—which is capable of exerting a powerful bacteriolytic action on many organisms, pathogenic and non-pathogenic. As a result of the above considerations it was decided to carry out a series of experiments to compare the antigenic properties of a vaccine killed by heat with those of a similar vaccine which had been dissolved by a lysozyme-containing substance, viz. egg-white.

TECHNIQUE.

The preparation of the vaccines.—The organism selected was a fæcal streptococcus, which was very susceptible to the action of lysozyme. A suspension of the organism in normal salt solution containing 1000 million streptococci per c.c. was completely dissolved by egg-white, diluted 1 in 3000, and by human tears, diluted 1 in 1000, after incubation at 45° C. for 18 hours.

The vaccine was prepared by washing off two 24-hour agar plate-cultures into 10 c.c. of normal saline. This suspension was counted and diluted to give a content of 10,000 million organisms per c.c.; the suspension was then divided into two equal parts. The first part was

killed by heating at 60° C. for 1 hour, while to the second half was added tears to make a concentration of 1 in 10, and this was incubated at 42° C. for 24 hours; this latter treatment sufficed to dissolve all the organisms, as was shown by attempting to stain a film of the dissolved organisms by Gram's method, when nothing was seen microscopically except a trace of amorphous Gram-negative *débris*; the importance of the complete solution of the organisms will be pointed out later. Both vaccines were suitably diluted for the injection of varying doses into rabbits.

Preliminary experiments and technique.—For the purpose of the experiment six rabbits of approximately the same age and weight were chosen, two (A, B) to receive injections of the ordinary vaccine, two (C, D) to receive injections of the dissolved vaccine, and two (E, F) to act as controls. As a preliminary the blood-sera of all the rabbits were tested against the test streptococcus to ascertain whether there were any natural immune bodies present.

The description of the technique which follows under the headings of the various tests is the same as that used throughout the experiment.

1. *Bacteriolysis.*—Two-fold serial dilutions of the fresh sera in normal saline were made in 1 c.c. quantities in small test-tubes; one drop of a thick suspension of the streptococcus in saline was added to each tube and to control volumes of saline. The tubes were incubated at 45° C. and readings were taken after 3 hours and 18 hours. There was no visible lysis after 3 hours, and after 18 hours only a trace of lysis was evident in all dilutions of the various sera up to 1 in 20. This was repeated on several occasions to see whether there was normally any variation in the bacteriolytic titre; a very slight variation took place in two of the sera, the titres of the others remaining constant.

2. *Bactericidal power:* (a) *Serum.*—Serial two-fold dilutions of the sera were made in normal saline on paraffined slides, using volumes of 10 c.mm., commencing with 10 c.mm. of undiluted serum and diluting up to 1 in 128, with a control volume of 10 c.mm. of saline; 10 c.mm. of a 24-hour broth culture of the streptococcus diluted 1 in 500,000 was added to each volume, and the mixtures were then taken up separately in pipettes, which were sealed and incubated for one hour at 37° C. The volumes were then blown out on to separate sections of agar plates divided into eight sectors and spread; the plates were incubated overnight at 37° C. and the discrete colonies in the different sectors were then counted. The sera of all the rabbits had a fairly well-marked bactericidal action on the streptococcus up to a dilution of serum of 1 in 16 at the commencement of the experiment. (b) *Defibrinated blood.*—For these tests the slide-cell method, as described by Sir A. E. Wright (1923), was used. The cells consisted of two sections instead of the usual four, and the bactericidal mixtures consisted of 100 c.mm. fresh defibrinated blood implanted with 25 c.mm. of a 24-hour broth culture of the streptococcus diluted 1 in 1,000,000 and 1 in 500,000; the blood was also diluted 1 in 2 in saline and implanted with the same suspensions. Controls consisted of (i) 25 c.mm. of the diluted broth culture seeded on agar plates and the colonies counted after growth, or (ii) 50 c.mm. blood mixed with 25 c.mm. of the diluted broth culture and 50 c.mm. of a 1 per cent. solution of saponin, which destroys the bactericidal power of the blood and allows the organisms to grow unrestricted in a slide-cell. This technique was used several times during the experiments, but was not used in the preliminary tests.

3. *Agglutinins.*—A serial two-fold dilution of the serum was made commencing at 1 in 10 and using 1 c.c. volumes, and one drop of a suspension of the organism in saline from a 24-hour agar slope culture standardized arbitrarily by opacity was added to each tube; readings were taken after three hours' incubation at 55° C. and after lying on the bench overnight. The streptococcus was not agglutinated by any of the rabbits' sera at the commencement of the experiment.

4. *Precipitins.*—The presence of precipitins in the sera was tested during the experiments, using three different precipitinogens, prepared as follows:

a. A broth culture of the streptococcus 7 days old was filtered through a Berkefeld filter and the sterile filtrate was used as a precipitinogen.

b. The clear supernatant from a centrifuged saline suspension of the organism which had been sealed off in a test-tube and allowed to autolyse at 37° C. for 4 weeks.

c. A 24-hour broth culture (100 c.c.) of the streptococcus was killed by heating to 60° C. for one hour; 10 c.c. egg-white were then added and the mixture was incubated at 42° C. for 24 hours to obtain complete solution of the organisms. This almost clear suspension was used as precipitinogen.

The sera were tested by making serial dilutions of the various precipitinogens in 1 c.c. volumes and adding to each tube of a series 100 c.mm. of the serum; the results were read after 6 hours at room temperature.

5. *Opsonins.* – The opsonic power of the sera was tested in the usual manner, normal serum and leucocytes being obtained from control rabbits; the suspension of organisms consisted of a small loopful of an 18-hour agar culture of the streptococcus in 1 c.c. of normal saline. The opsonic mixtures were incubated for 15 minutes at 37° C., and then films were prepared and examined. The opsonic power of the sera was not tested in the preliminary experiments.

THE ANTIBODY RESPONSE TO INJECTION OF VACCINES, LYSOZYME-DISSOLVED AND HEAT-KILLED.

The preliminary experiments having shown that the sera of the rabbits possessed only a slight bacteriolytic and bactericidal action on the streptococcus, the test rabbits were given intravenous injections of the vaccines as shown in the following table. Rabbits A and B were given inoculations of heat-killed streptococci, and rabbits C and D were given lysozyme-dissolved streptococci throughout the experiment, while rabbits E and F were kept as controls. The various immunity tests were carried out weekly between the doses of vaccines.

TABLE I.—*To show Dosage of Vaccines Inoculated into Test Rabbits.*

Date.	No. of organisms inoculated.	Date.	No. of organisms inoculated.
3.9.24	5 million.	4.10.24	500 million.
13.9.24	50 „	11.10.24	1000 „
20.9.24	100 „	18.10.24	2500 „
27.9.24	250 „	25.10.24	5000 „

Agglutinating Power.

The results of the weekly tests showed a formation of agglutinins in the sera of rabbits A and B which were inoculated with the heat-killed vaccine, while the sera from rabbits C and D, which were inoculated with the dissolved vaccine, showed no formation of agglutinins throughout the series of experiments; the sera of the control rabbits E and F remained negative throughout.

Attention may here be drawn to the fact emphasized above, *i.e.* the importance of the complete solution of the organisms in the vaccine treated with lysozyme, otherwise a slight formation of agglutinins may take place.

The results of the tests are shown in graphic form in Chart 1, which represents the averaged titres of each pair of test rabbits.

An example of a test carried out during the eighth week of the experiment is shown in the following table:

Agglutinating Power of Sera.

No. of rabbit.	Dilution of serum 1 in—								
	40.	80.	160.	320.	640.	1280.	2560.	5120.	10,240.
A	+++	+++	+++	++	++	++	+	+	tr.
B	+++	+++	+++	++	++	++	+	+	—
C, D, E and F	—	—	—	—	—	—	—	—	—

Bacteriolytic Power.

The bacteriolytic power of the sera showed a gradual and steady increase in the case of rabbits A and B (heat-killed vaccine). Except for a slight variation on two occasions (Chart 2), the sera from rabbits C and D (dissolved vaccine) showed no change in titre, while the titres of the control sera E and F also remained constant. The following table illustrates a typical result obtained in a test done towards the end of the series of experiments:

Bacteriolytic Power of the Sera. (18 hrs. at 45° C.)

No. of rabbit.	Dilution of serum 1 in—					
	10.	20.	40.	80.	160.	320.
A (heat-killed)	AC	±	±	±	tr.	tr.
B „	AC	±	±	±	tr.	—
C (dissolved vaccine) . . .	±	tr.	—	—	—	—
D „ „ . . .	±	tr.	—	—	—	—
E (control)	±	tr.	tr.	—	—	—
F „	tr.	tr.	—	—	—	—

AC indicates almost complete lysis.
± „ lysis well-marked.
tr. „ trace of lysis.

Bactericidal Power.

The results of this series of tests are shown graphically in Chart 3, and it will be seen that there was a steady and almost coincident increase in the bactericidal power of the sera, both of the rabbits inoculated with the heat-killed vaccine, and of those inoculated with the lysozyme-dissolved vaccine; the sera of the control rabbits showed no variation in bactericidal power.

The sera were also tested after heating to 56°C. for 30 minutes in order to see how much, if any, of the bactericidal action was due to lysozyme in the rabbits' serum. It was found that the heated sera were bereft of bactericidal power, and it was therefore concluded that the bactericidal action of the sera was probably not due to lysozyme, as the latter is not destroyed by heating to 56°C. for 30 minutes.

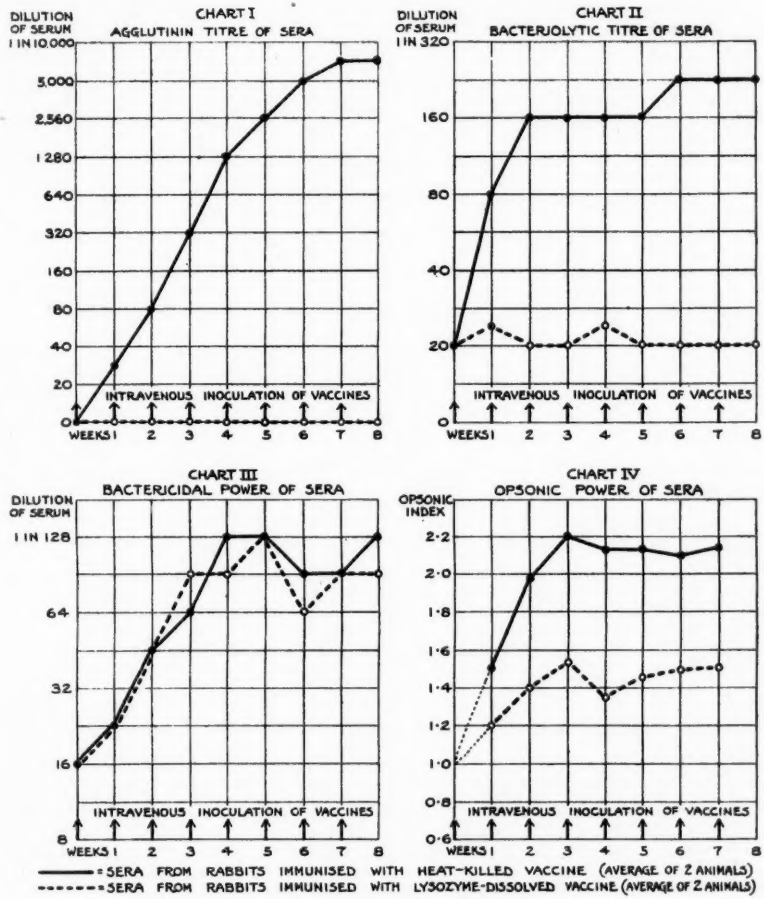
The following table shows the count of colonies on a series of plates, in an experiment carried out during the last week of the test; the end-point of the bactericidal power is starred in each case:

Bactericidal Power of Sera.

No. of rabbits.	Dilution of serum.							
	2.	4.	8.	16.	32.	64.	128.	Control.
A (heat-killed vaccine)	23	32	36	65	106	140	175*	209 colonies
B „ „	39	49	71	83	124	165*	203	217 „
C (dissolved vaccine) .	34	53	69	75	106	108	149*	201 „
D „ „ .	46	63	81	99	119	153*	180	206 „
E (control) . .	68	84	104	132*	173	191	181	198 „
F „ . .	63	91	119	148*	186	207	218	211 „

The bactericidal power of the whole blood (defibrinated) was also tested on several occasions, using the slide-cell technique already described; blood from

three of the rabbits was tested in each experiment, and the following tables show the results obtained in two of the experiments, which included the whole six rabbits.



Bactericidal Power of the Whole Blood (I).

No of rabbit.	24-hour broth culture diluted 1 in— 1,000,000. Dilution of blood. 1.	½.	Control.	500,000. Dilution of blood. 1.	½.	Control.
A (heat-killed vaccine)	32	50	82 colonies	46	96	155 colonies
C (dissolved vaccine) .	14	38	79 ,,	21	93	163 ,,
F (control) . . .	53	65	84 ,,	82	110	160 ,,

Bactericidal Power of the Whole Blood (II).

No. of rabbit.	24-hour culture diluted 1 in—					
	1,000,000. Dilution of blood.			500,000. Dilution of blood.		
	1.	½.	Control.	1.	½.	Control.
B (heat-killed vaccine)	22	36	51 colonies	38	70	109 colonies
D (dissolved vaccine) .	9	29	56 „	30	62	114 „
E (control) . .	37	46	59 „	57	91	112 „

The results confirm those obtained with the sera, and also show that, taking E and F, the controls, as normal, the killing power of the blood from rabbits C and D (dissolved vaccine) is slightly greater than that from rabbits A and B (heat-killed vaccine).

Opsonic Power.

In this series of tests (see Chart 4) there was a gradual and well-marked increase in the opsonic power of sera A and B (heat-killed vaccine), while sera C and D (dissolved vaccine) showed a concurrent but much less marked increase. The opsonic power of the sera was also tested after the latter had been heated to 55° C. for 30 minutes, using in the opsonic mixture double the dose of organisms which had been used for the unheated sera. In the earlier experiments the opsonic mixtures were incubated for 15 minutes at 37° C. before making films. It was noticed, however, that many of the phagocytes in all the mixtures contained organisms which had the usual characteristics of intracellular digestion, being often very much enlarged, staining very faintly, and showing evidence of disintegration. Probably owing to its susceptibility to lysozyme, this organism was found to undergo very rapid intracellular digestion in the phagocytes; the incubation period of the opsonic mixtures was therefore decreased to 6 minutes at 37° C., and the results showed that the increase in the opsonic power of sera A, B, C and D was greater after incubation for 6 minutes than after incubation for 15 minutes. This was probably due to the fact that incubation for 15 minutes allowed intracellular digestion of many of the organisms to be completed, and they were thus missed in the opsonic count. The increase in the opsonic index of the sera of the four test rabbits as compared with the controls, after decreasing the incubation period, was probably due to the fact that a larger amount of phagocytosis per leucocyte had occurred in the test sera during 15 minutes' incubation, and therefore a larger amount of intracellular digestion, than in the normal sera.

Chart 4 represents the opsonic powers of the unheated sera after incubation for 15 minutes at 37° C.

Precipitins.

The sera tested for the presence of precipitins gave negative results, and no evidence of precipitin formation was found at any time during the course of the experiments. This is in accordance with the known fact that precipitin formation is often not demonstrable as an immunity reaction in experiments with streptococci.

Complement-fixation.

A series of experiments was carried out to test the power of the sera of the rabbits to deviate complement. The technique employed was similar to that described by Fleming (1909) for carrying out the Wassermann test with minimal amounts of serum.

The antigens used were (1) a suspension in saline of living streptococci, of the same strain as used in the vaccines; (2) a control antigen, consisting of a suspension of *Staphylococcus aureus* in saline.

The result of this experiment showed a very slight but negligible difference in complement-fixing power in favour of the sera of rabbits A and B, *i. e.* those immunized with the ordinary heat-killed vaccine.

Precipitin formation by M. lysodeikticus.

As has already been shown, no formation of precipitins was demonstrable in the sera of any of the rabbits immunized with the test strain of *Streptococcus fæcalis*, dissolved or undissolved. Another experiment was therefore carried out similar in all respects to the above, using a different organism. The organism selected was *M. lysodeikticus*, which, as has been shown in previous papers, is very susceptible to lysozyme action, and has been regularly used as indicator organism in testing for the presence or absence of lysozyme in various tissues and secretions.

A thick suspension of *M. lysodeikticus* in saline was prepared, and diluted to contain 20 thousand million organisms per c.c.; the vaccine was divided into two equal parts, one-half being dissolved by tears, while the other was killed by heat. The vaccines were injected intravenously into rabbits as before, commencing with a dose of 5000 million organisms, increasing at intervals of three days up to a maximum dose of 25,000 million organisms. A fortnight later the rabbits were bled, and the sera were tested for the presence of precipitins, using as precipitinogens preparations of the organism as already described with the fæcal streptococcus.

Rabbit N (immunized with a vaccine of heat-killed *M. lysodeikticus*.

	Dilution of precipitinogen 1 in—								
	2.	4.	8.	32.	256.	512.	1024.	2048.	4096.
(1) Broth filtrate .	++	+	+	tr.	—	—	—	—	—
(2) Centrifuged autolysate .	+++	++	+	tr.	—	—	—	—	—
(3) Dissolved suspension .	—	++	+++	+++	+++	++	+	tr.	—

Rabbit J.—(Immunized with a vaccine of tear-dissolved *M. lysodeikticus*). No evidence of precipitin-formation in any of the three series.

Rabbit K (Control).—*Nil* in all the three series.

The heat-killed vaccine of *M. lysodeikticus* has therefore stimulated the formation of precipitins to a low titre (1/32) as tested with the broth filtrate and the autolysate, but the tear-dissolved suspension has acted as a much more sensitive and efficient precipitinogen, giving a titre of 1 in 2048; the absence of the reaction in the stronger dilutions of the tear-dissolved precipitinogen was concluded as being due to a zone of no reaction.

The dissolved vaccine has not stimulated the formation of precipitins, and subsequent tests showed a complete absence of formation of agglutinins and bacteriolysins, while the serum from the rabbits immunized with the heat-killed vaccine gave an agglutination titre of 1 in 500, and showed an increase in the bacteriolytic titre.

DISCUSSION.

Other workers have carried out experiments of a nature similar to those described above, but most of the work done has been with a view to determining whether the cleavage products of certain toxin-producing organisms were still toxic when injected into animals.

Douglas and Fleming (1921) in some experiments carried out with organisms digested with trypsin, subsequent to extraction with acetone, found on injection into animals a very much decreased power to stimulate the formation of agglutinins as compared with the untreated organisms, while the bactericidal power was increased equally as or more than that resulting from inoculation with untreated organisms. Continuing these experiments with reference to the antigenic properties of organisms acetone-extracted and trypsin-digested, Douglas (1921) found, using *B. typhosus*, that injection of the digests into animals caused a production of bactericidins and precipitins much greater than those produced by injection of the organisms, dried or acetone-extracted only, but no formation of agglutinins or opsonins, and deduced that the formation of agglutinins and precipitins in the serum was stimulated by different phases in the solution of the organisms; he also suggested that antigens giving rise to agglutinins and opsonins were produced at an early stage of the solution of the organisms by the body-fluids, while bactericidins, precipitins and antitoxins were formed during a later period of the lytic process. The results of the present experiments agree substantially with those obtained by Douglas, except as regards the formation of precipitins.

The following table gives a comparison of the antibodies produced in Douglas's experiments and in those described above:

Author	Douglas.		Allison.			
Organism	*B. typhosus.*		*S. fæcalis.*		*M. lysodeikticus.*	
Nature of vaccine.	Dried organisms.	Acetone-extracted and trypsin-digested.	Heat-killed.	Lysozyme-dissolved.	Heat-killed.	Lysozyme-dissolved.
Bacteriolysins	—	—	++	—	+	—
Precipitins	+	++	—	—	++	—
Bactericidins	+	++	++	++	Not done	Not done
Opsonins	+++	+	++	+	,,	,,
Agglutinins	+++	—	+++	—	++	—
Complement-fixing substances	Not done	Not done	+	+	Not done	Not done
Antitoxin	++	++	Non-toxic	Non-toxic	Non-toxic	Non-toxic

In the present experiments an increase in the bacteriolytic power of the serum was noted in the case of animals inoculated with the ordinary vaccine, while Douglas described a diminution of the lytic power after injection of untreated bacteria; the absence of lysins in the sera of the rabbits inoculated with dissolved *S. fæcalis* may have been due to the organism having been dissolved beyond the stage which stimulated the production of bacteriolysins.

If the theory of Douglas that the formation of agglutinins and precipitins in the serum is stimulated by different stages of solution of the organisms in the body-fluids is correct, the above experiments (see table) indicate that the precipitinogen stage of solution of the organisms, if formed later than the stage stimulating the formation of agglutinins, is produced at an earlier "phase" of solution than that which stimulates the formation of bactericidal and complement-fixing substances.

Dreyer (1923) in his experiments with "defatted" antigens also noted the absolute independence of agglutinins, precipitins, and complement-fixing substances in the serum, using as antigens "defatted" *B. anthracis* and "defatted" *B. typhosus*.

It has been pointed out that digests of bacteria act as excellent antigens in tests for the presence in serum of complement-deviating substances, and the above experiments with *M. lysodeikticus* show that the dissolved organism acts as a much more efficient and sensitive antigen in tests for the presence of precipitins than the commonly used salt autolysate.

CONCLUSIONS.

When organisms dissolved by a lysozyme-containing substance are injected into animals, there is a well-marked increase in bactericidal power, with formation of specific complement-fixing substances. There is also a slight rise in the opsonic power, but no increase in the bacteriolytic, precipitating or agglutinating powers.

These results agree in substance with those obtained by Douglas, using tryptic digests of acetone-extracted organisms, except as regards the formation of precipitins. This may be due to the lysozyme-dissolved organisms having been broken down beyond the precipitinogen stage of solution.

The presence or absence in the sera of the various immune bodies, after inoculation with lysozyme-dissolved organisms, agrees with the theory of Douglas that the antigens stimulating the formation of agglutinating and opsonic powers are produced during the early stage of the lysis of the organisms by the body-fluids, while the antigens giving rise to the formation of precipitins, though produced later, are formed earlier than those stimulating the formation of bactericidal and complement-fixing substances.

The absence of formation of bacteriolysins in the serum, after inoculation with dissolved organisms, compared with the increase observed after inoculation of untreated organisms, seems to indicate that the formation of bacteriolysins is stimulated by an early split product of the organisms. This is contrary to Douglas's view, which suggested that the lytic powers are developed at a later stage of solution than that stimulating the formation of agglutinins and opsonins.

Organisms dissolved by lysozyme acted as a much more effective and sensitive antigen, in testing for the presence of precipitins, than a salt autolysate of the same organisms, or the supernatant from a broth culture, seven days old.

In conclusion I should like to thank Dr. Alexander Fleming for much helpful advice and criticism during the course of the experiments.

REFERENCES.

DOUGLAS, S. R.—(1921) *Brit. J. Exper. Pathol.*, **2**, 175.
DOUGLAS, S. R., AND FLEMING, A.—(1921) *Ibid.*, **2**, 131.
DREYER, G.—(1923) *Ibid.*, **4**, 146.
FLEMING, A.—(1909) *Lancet*, **1**, 1512.—(1922) *Proc. R. Soc.*, B, **93**, 306.
FLEMING, A., AND ALLISON, V. D.—(1923[1]) *Ibid.*, B, **94**, 142.—(1923[2]) *Brit. J. Exper. Pathol.*, **3**, 259.—(1924) *Lancet*, **1**, 1303.
WRIGHT, SIR A. E.—(1923) *Ibid.*, **1**, 365, 417, 473.

NON-SPECIFIC STIMULATION OF ANTIBODIES: THE EFFECT OF MANGANESE ON AGGLUTININS.

E. S. HORGAN.

From the Department of Bacteriology, Trinity College, Dublin.

Received for publication March 21st, 1925.

CONSIDERABLE attention has been paid in recent years to non-specific protein therapy, and also to non-specific stimulation of antibodies. The present paper deals only with the latter, but it is possible that some connection exists between the two subjects.

The most striking results have been obtained in Copenhagen. It was found that the yield of diphtheria antitoxin in horses could be considerably raised by the intravenous injection of various metallic salts, of which manganese and beryllium gave the best effects. All antitoxin-producing horses at the Danish State Serum Institute are now given manganese, and it is claimed that the average yield of an animal is considerably increased.

Similar experiments have been carried out on the stimulation of agglutinins and hæmolysins in goats and rabbits, and on the bactericidal power of the blood in goats (Walbum, 1923[1,2]). Walker (1920) has also found that salvarsan appears to induce fresh and prolonged activity in the

production of typhoid agglutinins in rabbits. On the other hand, McIntosh and Kingsbury (1924) found completely negative results in somewhat similar experiments. Mackie (1924) obtained very variable actions in his attempts to stimulate antibodies by manganese and beryllium, some animals showing increases, while others were negative or even showed decreases. Pico (1924) was able to raise the titre of diphtheria antitoxin in horses but not that of tetanus by the administration of manganese. The metal had no effect on agglutinins, precipitins or hæmolysins.

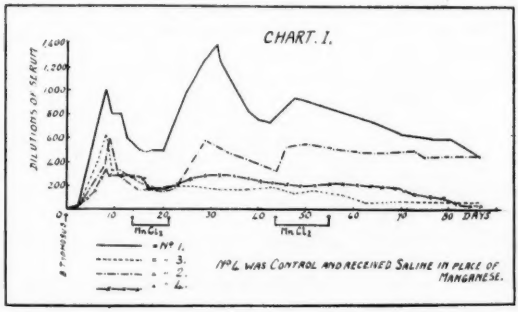
The results of the above workers are contradictory, and no explanation has so far been suggested of the superiority of the Danish results.

In the following experiments an endeavour has been made to ascertain the effects of intravenous injection of manganese on the production of agglutinins in rabbits.

The rabbits received approximately one hundred million *B. typhosus* vaccine intravenously and the agglutinin content of the serum was in most cases estimated daily. Dreyer's method (1920) was used, and the same bacterial emulsion throughout the series both for agglutination and for the intravenous injections in suitable dilutions. As the ordinary method of doubling the dilutions might miss slight changes, intermediate dilutions were made in every case.

It is well known that following a severe hæmorrhage the antibody content of serum is increased, and Hartley (1924) has found that even small bleedings may have the same effect. To avoid this as far as possible only small quantities of blood were taken (0·5 to 1 c.c.), and as far as can be seen the results were not materially affected by such bleedings.

Chart I shows the curves of four rabbits. All four gave approximately the normal type of curve, the primary peak in each case being at the seventh day. When the curves had dropped to a comparatively constant level, the manganese was given to Nos. 1, 2 and 3, while No. 4 was kept as control, receiving in place of the manganese equivalent amounts of normal saline. Pure crystallized manganous chloride was used as a N/1000 solution in 0·85 per cent. NaCl, each animal receiving intravenously 1 c.c. per kilo body-weight (Walbum). The injections were given in two series with about six injections in a series, as after this number it was extremely difficult to find a patent vein, owing to the thrombosis caused by the manganese. However, after a few days' rest the veins became patent again, probably owing to canalization of the thrombi.



Rabbit No. 1 showed the highest primary peak and also the best response to the manganese; No. 2 showed similar but slighter changes; No. 3 was

completely negative, and indeed fell with the second series of injections; while the control (No. 4) kept a practically constant curve.

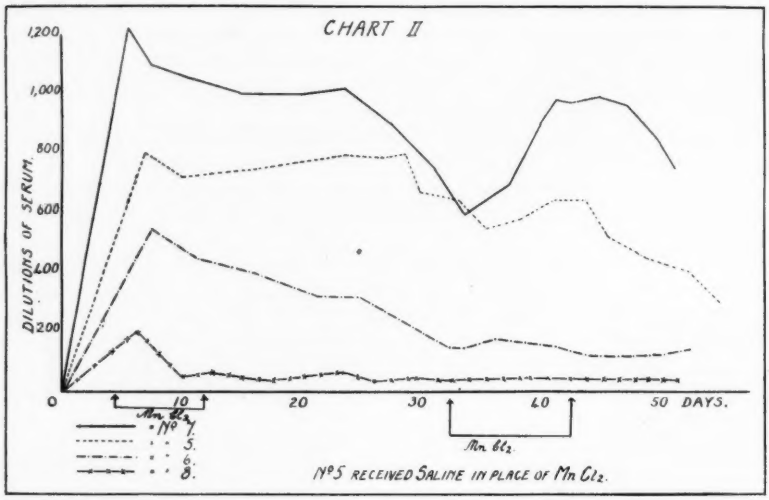
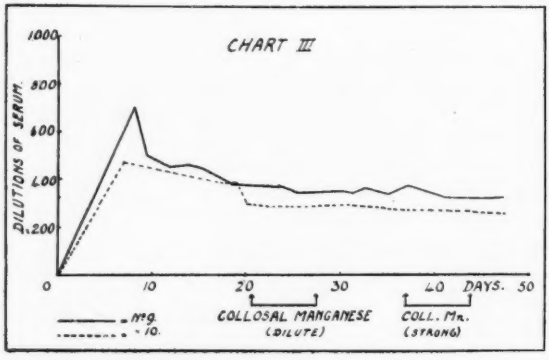


Chart II.—Rabbits Nos. 6, 7, 8, with No. 5 as a control:

The first series of injections was given when the primary peaks were about their highest. The curves did not appear to be influenced in any way, as they fell in the normal manner. The second series showed a fairly well-marked rise in No. 7, but no change in the others; control (No. 5) showed a slight rise. In this connection Walbum claims that saline has a slight stimulating action on the agglutinin curve, which he ascribes to the sodium atom.

Chart III.—Rabbits Nos. 9 and 10:



In place of the chloride, collosol manganese (Crookes) was employed in two series, first in a dilution corresponding to an equivalent amount of $MnCl_2$. As these doses had no effect, the strong collosol was used in doses of 0·5 to 1 c.c. for each animal. No difference could be observed in the curves, nor did these large doses appear to have any toxic effects.

DISCUSSION.

Manganous chloride appeared to stimulate the agglutinin production in 3 only out of 6 animals, and it will be seen from the charts that all three had well-marked primary peaks also. The degree of stimulation was slight (greatest 1/500 to 1/1400). The manganese had apparently no effect in preventing the normal fall from the primary peak. Collosol manganese had no effect in either animal on which it was used.

Sex did not appear to make any difference, Nos. 1, 4 and 6 being females, of which the control (No. 4) became pregnant during the investigation. Walker found in one of his animals that pregnancy seemed to produce a curious, rather prolonged stimulating effect on the agglutinin production. In this present case pregnancy made no difference to the curve.

The results on the whole are disappointing, but appear to correspond somewhat with Mackie's observations. There is possibly some connection between the potentiality of an animal to produce antibodies and the power of response to the non-specific stimulation of manganese.

CONCLUSIONS.

(1) Manganese does not prevent the normal fall in agglutinating titre from the primary peak. In 3 out of 6 animals, when the injections were given after the primary fall, the chloride caused a slight rise in titre, the maximum being nearly threefold.

(2) These three animals had all well-marked primary peaks.

(3) Collosol manganese gave negative results, the curves falling as in the controls.

(4) The suggestion is made that there is some connection between the potentiality of the animal to produce antibodies and the power of response to the non-specific stimulation of manganese.

I wish to thank Prof. J. W. Bigger for his advice and assistance throughout this investigation.

REFERENCES.

WALBUM, L. E.—(1923[1]) *Ann. de l'Inst. Pasteur*, **38**, 396.—(1923[2]) *C.r. Soc. de biol.*, **89**, 1007.

WALKER, E. W. AINLEY.—(1920) *Med. Res. Council, Spec. Rep. Ser.*, No. 55. London (H.M. Stationery Office).

MCINTOSH, J., AND KINGSBURY, A. N.—(1924) *Brit. J. Exper. Pathol.*, **5**, 18.

MACKIE, T. J.—(1924) *Brit. Med. J.*, **2**, 1100.

PICO.—(1924) *C.r. Soc. de biol.*, **90**, 1049.

HARTLEY, P.—(1924) *Brit. J. Exper. Pathol.*, **5**, 306.

DREYER, G.—(1920) *Med. Res. Council, Spec. Rep. Ser.*, No. 51. London (H.M. Stationery Office).

THE ANTIGENIC PROPERTIES OF PRECIPITATES PRODUCED BY THE INTERACTION OF DIPHTHERIA TOXIN AND ANTITOXIN.

PERCIVAL HARTLEY.

From the National Institute for Medical Research, Hampstead, London.

Received for publication April 17th, 1925.

THE active immunization of human beings against diphtheria by means of slightly toxic mixtures of toxin and antitoxin has been practised for some years. The mixtures contain, in addition to the valuable immunizing agent, quantities of non-specific poisonous substances which are frequently the cause of unpleasant local reactions in inoculated individuals. These arise from the culture medium used for the preparation of the toxin, the products of bacterial growth other than toxin, bacterial protein and horse-serum. Up to the present time all attempts to remove the undesirable constituents from the valuable diphtheria prophylactic have failed.

The specific toxicity of toxin-antitoxin mixtures can be controlled by antitoxin and by means of certain chemicals. Glenny and his co-workers (1921–1923), in their attempts to prepare an ideal immunizing agent, have shown that the specific toxicity of diphtheria toxin can be enormously reduced by treatment with formalin, and that such modified toxin—consisting, in fact, almost entirely of toxoid—is a valuable immunizing agent, used either alone or in conjunction with small amounts of antitoxin. Ramon (1923) has also used formalin-treated toxin for the immunization of horses and human beings and has obtained excellent results. Schroder and Park (1923) found that old, deteriorated toxin gave a fairly satisfactory immunity response and produced little local reaction. All these preparations, however, still contain the non-specific poisonous substances, and, in view of the wide and increasing use of diphtheria prophylactic, the discovery of a process which will remove the undesirable elements and supply the active immunizing agent in a more or less pure form is an urgent practical need.

The recent investigations of Ramon (1922–1924) suggested that the problem of eliminating the non-specific poisonous substances from diphtheria prophylactic might be attacked from another angle. Ramon has shown that neutral, or nearly neutral, mixtures of toxin and antitoxin flocculate on standing at room temperature. During an investigation of the properties of this precipitate carried out in this Institute it was found that the well-washed precipitate from such mixtures was harmless to guinea-pigs, and, moreover, that the animals so injected developed a certain amount of immunity to diphtheria toxin. This result suggested that the precipitate contained toxin and antitoxin, and this was subsequently proved to be the case, in later experiments by Ramon (1923), who showed that the precipitate contained

about three-quarters of the antitoxin units present in the original mixture. The precipitate is apparently quite insoluble in normal saline; thorough washing with this fluid removes practically all the non-specific poisonous substances in the mixture and a white insoluble precipitate is obtained, which, as stated above, possesses antigenic properties.

Ramon has established the interesting fact that the precipitate produced in a toxin-antitoxin mixture has the same specific properties as the fluid from which it separates, *i. e.* it is either toxic, neutral, or antitoxic, according as the mixture is under-neutralized, exactly neutralized, or over-neutralized with antitoxin. It was of interest to see whether the antigenic properties of toxin-antitoxin precipitates varied with the nature of the mixture in which they were produced. If this proved to be the case, then the preparation of a relatively pure antigen for diphtheria prophylaxis would become a possibility.

In the experiments described below precipitates have been prepared from mixtures containing varying amounts of antitoxin, and their antigenic properties have been investigated. An unexpected difficulty arose in connection with the preparation of certain of these precipitates. Old mature toxins, suitable in all respects for the preparation of toxin-antitoxin mixtures of the usual type, have been used. These, as well as the antitoxin, were accurately standardized, and many experiments have been carried out with them. It has been found that mixtures which contain one or more units of antitoxin to every L_+ dose of toxin flocculate unless the antitoxin is in very large excess. If the excess of antitoxin is small, precipitation is slow and the amount of precipitate produced is small. If the proportion of antitoxin is less than 1 unit for every L_+ dose of toxin the mixtures remain clear. These results differ from those obtained by Ramon, who found that the washed precipitate which separated from a definitely toxic mixture was lethal for guinea-pigs. As Ramon's later work shows, the difference is due to the fact that he uses fresh toxins, while in these experiments old mature toxins were used. This failure to obtain precipitates from mixtures which were of the requisite composition and toxicity for use in practice* has somewhat limited the scope of the present series of experiments, as it was hoped to include a precipitate from a mixture which contained a small amount—or even a definite excess—of unneutralized toxin.† The results obtained with the precipitate from Mixture 7 (Experiment 4) suggest that the antigenic value of such a preparation would be greater than any of those investigated in the present series of experiments.

Glenny, Allen and Hopkins (1923) have described methods for testing the antigenic value of toxin-antitoxin mixtures. These workers consider that the toxicity test alone may lead to incomplete or erroneous conclusions as to the immunizing value of a preparation. They use rabbits and guinea-pigs, and measure, in a variety of ways, the immunity response following injection of the material under investigation. One of their methods is very simple and has been used in their more recent work. It is carried out as follows: Guinea-

* The writer is informed that such mixtures do occasionally flocculate, but that this is not usual. (Personal communication from Mr. A. T. Glenny and Dr. A. T. MacConkey.)

† Since the above was written mixtures of toxin (freshly prepared) and antitoxin have been made which are lethal for guinea-pigs in small doses and also flocculate readily. The precipitates are toxic, as Ramon found, and their antigenic properties are now being investigated.

pigs are injected with a single dose of toxin-antitoxin mixture, and three or four weeks later the animals are inoculated intracutaneously with a Schick test dose of diphtheria toxin. If the animals react negatively the "immunity index" is said to be 1. If the animals react positively a second Schick test dose is given a week later and the weekly injections are continued until the reaction is negative. The "immunity index" is defined as the number of such injections, given at weekly intervals, required to render the animal Schick-negative. If the antigenic value is low many weekly injections are needed. If the material is entirely devoid of antigenic value, the number will be the same as for the normal, uninoculated, control animals. Glenny and Hopkins (1923) have shown that in such normal animals the number of injections required to raise the immunity to the Schick-negative level is large, viz. 31 injections for a fresh toxin and 12 for an old, mature toxin. They found that with formalin-treated toxin the immunity index was occasionally as low as 1, and very frequently no greater than 2. This method has been used in the first three experiments described below.

Experiment 1 was purely qualitative in character, and was carried out to ascertain whether the precipitate obtained from toxin-antitoxin mixtures possessed any antigenic value at all. The precipitate from a number of tests carried out by Ramon's method, in which about 200 c.cm. of toxin were used, was collected and washed ten times with normal saline. The white insoluble precipitate was finally emulsified in 20 c.cm. of saline, and doses of 4 c.cm., 2 c.cm. and 1 c.cm. were injected into guinea-pigs, two animals being tested at each dose. The animals showed no ill-effects of the injection, even of the largest doses. The material was absorbed in about 24 hours, and the animals remained in good condition and increased in weight. The immunity index was determined, the first Schick test being made 4 weeks after the injection. The immunity index was 4 for each of the animals which received 4 c.cm. of precipitate-emulsion, 3 for each animal injected with 2 c.cm., and 4 and 3 for the animals which received 1 c.cm. Three normal uninoculated guinea-pigs were included as controls, and were given, at weekly intervals, 14 Schick test doses of diphtheria toxin. The positive reaction was just as marked after the fourteenth dose as after the first. The control animals developed no immunity, while the experimental animals, which had received the precipitate from toxin-antitoxin mixtures as primary stimulus, all developed a certain amount of immunity.

Experiment 2.—In this experiment an attempt was made to compare the antigenic value of the precipitate from a toxin-antitoxin mixture with that of the fluid remaining after this precipitate had been separated.

The preparation of the material for this experiment was based on the result of a flocculation test, carried out by Ramon's method, with the toxin and antitoxin used. This test showed that precipitation occurred first, in point of time, in the mixture consisting of 20 c.cm. toxin (L_+ dose = 0·20 c.cm.), and 0·275 c.cm. antitoxin (500 units per c.cm.). Accordingly 100 c.cm. of toxin and 1·375 c.cm. of antitoxin were mixed and allowed to stand at room temperature, in the dark, for 2 days. The toxin-antitoxin ratio in this mixture was 1 L_+ dose of toxin to 1·37 units of antitoxin. The clear supernatant liquid was pipetted off and, in order to remove any gross floating

particles, was filtered twice through filter-paper. The precipitate was washed ten times in a centrifuge tube with normal saline, and finally emulsified in 100 c.cm. of saline. Guinea-pigs were inoculated subcutaneously with doses of supernatant fluid and precipitate-emulsion as shown in Table I. For purposes of comparison the immunity index of two toxin-antitoxin mixtures (B.671 and B.870), prepared in the usual way for use in practice, was determined, and two uninoculated animals were included as controls.

The results of this experiment are given in Table I:

TABLE I.

Preparation injected.	Dose (c.cm.).	No. of animals tested.	Immunity index.
Precipitate from toxin-antitoxin Mixture 1	0·3	2	> 7, 5
	0·6	2	4, 4
	1·0	2	5, 7
	2·5	2	> 7 (1 died)
	5·0	2	4, 4
Fluid remaining after removal of precipitate from above Mixture 1	0·3	2	7, 5
	0·6	2	> 7, 7
	1·0	2	> 7, > 7
	2·5	2	> 7, 7
	5·0	2	7, 4
Toxin-antitoxin Mixture B.681	1·0	3	> 7, 3, 5
Toxin-antitoxin Mixture B.870	1·0	3	4, 3, 3
Controls	*nil*	2	> 7, > 7

Examination of this table shows that of the 9 surviving animals which received precipitate as primary stimulus, 4 gave negative reactions at the fourth test, 2 at the fifth test, and 1 at the seventh test. Two still gave positive reactions at the seventh test. On the other hand, of the 10 animals which received the supernatant fluid as primary stimulus, 1 gave a negative reaction at the fourth test, 1 at the fifth test, 4 at the seventh test, and 4 were still Schick-positive at the seventh test. The results suggest that the washed precipitate is a better antigen than the fluid which remains after this precipitate has been removed. The precipitate, however, is inferior to the toxin-antitoxin mixtures B.671 and B.870.

Experiment 3.—The L_+ dose of the toxin used in this experiment was 0·17 c.cm., the antitoxin being from the same sample as that used in the previous experiment. Three different mixtures were made, and in the case of two of these the antigenic values of (*a*) the whole mixture, (*b*) the precipitate and (*c*) the fluid remaining after removal of the precipitate were determined. The materials for this experiment were prepared as follows:

Mixture 2.—100 c.cm. toxin and 1·25 c.cm. antitoxin.
Ratio of toxin to antitoxin = 1 L_+ dose toxin : 1·06 unit antitoxin.
This mixture remained clear for some time and then became faintly opalescent. The opalescence persisted for 2 days, but no precipitation occurred. Two days after mixing

guinea-pigs were injected with 2 c.cm. and 1 c.cm. doses. Animals receiving the larger dose became paralyzed, and died in 27 to 30 days: those receiving 1 c.cm. quantities developed slight paralysis but recovered.

Mixture 3.—200 c.cm. toxin and 3 c.cm. antitoxin.

Ration of toxin to antitoxin = 1 L_+ dose toxin : 1·27 unit antitoxin.

This mixture became opalescent and by the following day precipitation had occurred. Guinea-pigs injected with 5 c.cm. and 2·5 c.cm. quantities remained well and survived.

170 c.cm. of the flocculated mixture were dealt with as described under Experiment 2, the well-washed precipitate being finally emulsified in 17 c.cm. of saline.

Mixture 4.—200 c.cm. toxin and 4·25 c.cm. antitoxin.

Ratio of toxin to antitoxin = 1 L_+ dose toxin : 1·80 unit antitoxin.

This mixture corresponds to the one in which, in the Ramon test, precipitation occurred first in point of time. The mixture was innocuous to guinea-pigs. The precipitate from 170 c.cm. was washed in the usual way and finally emulsified in 17 c.cm. of saline.

For purposes of comparison two animals were injected with toxin-antitoxin mixture B.671, and two uninoculated animals were included as controls. Further, in order to compare the amounts of material injected, the total nitrogen per c.cm. of the precipitate emulsion, of the fluid remaining after removal of the precipitate, and also of the toxin-antitoxin mixture B.671, was determined. These figures are included, with the other results of Experiment 3, in Table II.

There are many indications that the toxin used in Experiment 3 was particularly suitable for the preparation of toxin-antitoxin mixtures. Thus, Mixture 2, which approximates most closely to those prepared for general use, has an unusually low immunity index; yet, judged by the standard of toxicity adopted, even this preparation contains slightly too much antitoxin. Again, the antigenic value of the fluid remaining after removal of the precipitate (Mixtures 3 and 4) is considerable, in contrast with the result of the previous experiment. In this experiment it was assumed that precipitation was complete in 48 hours. This assumption, however, proved to be unwarranted, since it was observed that the clear fluid, separated after 48 hours, yielded a further quantity of precipitate after a few days, and this precipitation continued for some time. It is possible that the antigenic value of the supernatant fluid was due, in part at least, to some toxin-antitoxin complex remaining in solution at the time of testing and separating out as a precipitate later.

These results confirm those obtained in the preceding experiments; twenty guinea-pigs which were injected with varying doses of the washed precipitate from toxin-antitoxin mixtures all developed immunity to diphtheria toxin.

The figures showing the nitrogen content of the different preparations used in this experiment are of interest. Thus, two animals received 0·1 c.cm. of precipitate-emulsion, equivalent to the precipitate which separated from 1 c.cm. of Mixtures 3 and 4. The total nitrogen contained in these two doses was 0·021 mgm. and 0·026 mgm. respectively, and in each case the immunity index was 4. The immunity index for 2 c.cm. doses of B.671—a typical toxin-antitoxin mixture prepared in the usual way—was also 4, and in this case the material injected contained 4·48 mgm. of nitrogen. In other words, an amount of precipitate containing from 1/40th to 1/50th of a milligramme of nitrogen produced the same immunity response as a quantity of toxin-antitoxin mixture containing some 200 times as much nitrogen. The precipitate is

comparatively pure antigen: the mixtures prepared in the usual way consist mainly of extraneous foreign substances. Further confirmation of this result was obtained in Experiment 4.

TABLE II.

Preparation injected.	Dose. C.cm.	Dose. Total nitrogen (mgm.).	No. of animals tested.	Immunity index.
Toxin-antitoxin Mixture 2 $\frac{\text{Toxin}}{\text{antitoxin}} = \frac{1\ L_+\ \text{dose}}{1{\cdot}06\ \text{unit}}$	1·0	—	2	1, 2
Toxin-antitoxin Mixture 3 $\frac{\text{Toxin}}{\text{antitoxin}} = \frac{1\ L_+\ \text{dose}}{1{\cdot}27\ \text{unit}}$	5·0	—	3	2, 2, 2
	2·5	—	2	3, 3
Precipitate from toxin-antitoxin Mixture 3	0·1	0·021	1	4
	0·25	0·052	2	4, 3
	0·5	0·105	1	4
	1·00	0·21	2	4, 4
	2·5	0·525	2	3, 4
	5·00	1·05	1	3
Fluid remaining after removal of precipitate, Mixture 3	1·0	4·65	1	6
	2·5	11·62	2	2, 3
	5·0	23·25	1	5
Toxin-antitoxin Mixture 4 $\frac{\text{Toxin}}{\text{antitoxin}} = \frac{1\ L_+\ \text{dose}}{1{\cdot}8\ \text{unit}}$	5·0	—	3	2, 6, 2
	2·5	—	2	4, 3
Precipitate from toxin-antitoxin Mixture 4	0·1	0·026	1	4
	0·25	0·065	2	3, 4
	0·5	0·13	1	6
	1·00	0·26	2	4, 4
	2·5	0·65	2	3, 3
	5·00	1·3	1	3
Fluid remaining after removal of precipitate, Mixture 4	1·0	4·42	1	4
	2·5	11·05	2	4, 4
	5·0	22·1	1	3
Toxin-antitoxin Mixture B.671	2·0	4·48	2	4, 4
Controls—*nil*	*nil*	—	2	> 7, > 7

Experiment 4.—In this experiment the antigenic values of precipitates (prepared from three toxin-antitoxin mixtures of widely different composition) were compared by measuring, in another way, the immunity response which they evoked. The different preparations were injected into guinea-pigs, and after the lapse of 11 to 12 weeks the antitoxin produced was determined. About a week later each animal in the series was injected subcutaneously with a dose of toxin representing about three minimum lethal doses of toxin for guinea-pigs of the same weight. The results of this experiment show quite clearly that the antigenic value of a precipitate varies with the nature of the toxin-antitoxin mixture from which it separates.

The materials used in this experiment were prepared as follows:

Mixture 5.—200 c.cm. toxin (L_+ dose = 0·2 c.cm.) + 5·5 c.cm. antitoxin (475 units per c.cm.).

This mixture corresponds to the first flocculating tube in the Ramon test.

Toxin : antitoxin = 1 L_+ dose of toxin : 2·6 units antitoxin.

This mixture was innocuous to guinea-pigs in doses of 5 c.cm. After standing for 4 days the precipitate was separated, treated as described under Experiment 2, and finally emulsified in 20 c.cm. of saline.

1 c.cm. of precipitate-emulsion contained 0·266 mgm. of nitrogen.

Mixture 6.—170 c.cm. toxin (L_+ dose = 0·17 c.cm.) + 3·61 c.cm. antitoxin (500 units per c.cm.).

This mixture corresponds to the first flocculating tube in the Ramon test.

Toxin : antitoxin = 1 L_+ dose toxin : 1·8 units antitoxin.

After standing for 4 days, the precipitate was treated as above and emulsified in 17 c.cm. of saline.

1 c.cm. of precipitate-emulsion contained 0·21 mgm. of nitrogen.

Mixture 7.—(100 c.cm. of toxin (L_+ dose = 0·17 c.cm.) + 100 c.cm. saline) + 1·25 c.cm. antitoxin (500 units per c.cm.).

This mixture corresponds to the tube in the Ramon test which became faintly opalescent after 24 hours, but did not flocculate until the tenth day. Tested 24 hours after mixing, doses of 1 c.cm. produced paralysis with subsequent recovery in guinea-pigs, and doses of 2 c.cm. produced paralysis and death in about 3 weeks. This mixture, which corresponds closely to toxin-antitoxin mixtures prepared for general use, was allowed to stand at room temperature, in the dark, for 4 months. A fine precipitate, very small in amount, separated out very slowly, and settled to the bottom of the cylinder in which the mixture was stored. After 4 months the supernatant fluid was pipetted off, and the precipitate was thoroughly washed in the usual way and emulsified in 10 c.cm. of saline.

1 c.cm. of precipitate-emulsion contained 0·084 mgm. of nitrogen.

*Toxin-antitoxin mixture B.*671.—1 c.cm. of Mixture B.671 contained 2·24 mgm. nitrogen.

The results of this experiment are summarized in Table III.

The antigenic value of the precipitate from Mixture 5, in which the antitoxin was in large excess, is evidently very low. Twelve of the 14 animals failed to produce as much as a hundredth of a unit of antitoxin per c.cm. of serum, and all 12 succumbed to the injection of 3 M.L.D. of toxin. (This quantity of toxin killed 3 normal animals of about the same weight in 1½, 1½ and 2 days).

The precipitate from Mixture 6 gave surprisingly good results, having regard to the fact that in this case also the toxin was considerably over-neutralized. Ten of the 14 animals injected produced a hundredth of a unit, or more, and 10 of the 14 survived the injection of 3 M.L.D. of toxin.

The antigenic value of the toxin-antitoxin mixture B.671 appears to be about the same as that of the precipitate from Mixture 6. The antitoxin production in this series of eight animals was very variable, ranging from a

TABLE III.

Preparation injected.	No. of guinea-pig.	Dose. Volume (c.cm.).	Dose. Nitrogen content (m.gm.).	Antitoxin produced. Interval (days).	Antitoxin produced. Units per c.cm. serum.	Injection of diphtheria toxin. Interval (days).	Dose (c.cm.).	Local reaction.	Change in weight (grms.).	Result.
Precipitate from Mixture 5: Toxin / antitoxin = 1 L_+ dose / 2·6 units	1	0·1	0·0266	75	0·005	83	0·02	—	− 45	† 3½ days
	2	,,	,,	,,	< 0·005	88	,,	—	− 10	† 1¾ ,,
	3	,,	,,	,,	< 0·005	,,	,,	—	− 15	† 2 ,,
	4	,,	,,	,,	< 0·005	,,	,,	—	− 5	† 1¾ ,,
	5	0·5	0·133	83	< 0·002	,,	,,	—	− 10	† 1½ ,,
	6	,,	,,	,,	0·002	,,	,,	—	− 50	† 2 ,,
	7	,,	,,	,,	0·002	,,	,,	—	− 30	† 1½ ,,
	8	,,	,,	,,	0·002	,,	,,	—	− 50	† 2 ,,
	9	,,	,,	,,	0·002	,,	,,	—	− 35	† 1½ ,,
	10	,,	,,	,,	< 0·002	,,	,,	—	− 20	† 1½ ,,
	11	1·0	0·266	87	0·005	,,	,,	—	− 20	† 1½ ,,
	12	,,	,,	,,	0·005	,,	,,	—	− 20	† 1½ ,,
	13	,,	,,	,,	0·01	,,	,,	L.S.	— 5	Lived
	14	,,	,,	,,	0·02	,,	,,	*nil*	+ 40	,,
Precipitate from Mixture 6: Toxin / antitoxin = 1 L_+ dose / 1·8 units	15	0·1	0·021	75	0·01	83	0·02	M.S.	+ 15	Lived
	16	,,	,,	,,	0·02	88	,,	M.S.	0	,,
	17	,,	,,	,,	0·005	,,	,,	—	− 40	† 1½ days
	18	,,	,,	,,	0·005	,,	,,	M.S.	+ 10	Lived
	19	0·5	0·105	83	0·04	,,	,,	S.S.	+ 40	,,
	20	,,	,,	,,	0·014	,,	,,	V.L.S.	+ 30	,,
	21	,,	,,	,,	0·005	,,	,,	—	− 35	† 2 days
	22	,,	,,	,,	0·01	,,	,,	—	− 50	† 2½ ,,
	23	,,	,,	,,	0·02	,,	,,	M.S.	+ 20	Lived
	24	,,	,,	,,	0·005	,,	,,	—	− 20	† 1½ days
	25	1·0	0·21	87	0·01	,,	,,	L.S.	− 30	Lived
	26	,,	,,	,,	0·03	,,	,,	S.S.	0	,,
	27	,,	,,	,,	0·02	,,	,,	M.S.	− 20	,,
	28	,,	,,	,,	0·01	,,	,,	V.L.S.	− 20	,,
Precipitate from Mixture 7: Toxin / antitoxin = 1 L_+ dose / 1·06 units	29	0·1	0·0084	75	0·01	83	0·02	M.S.	+ 10	Lived
	30	,,	,,	,,	0·005	88	,,	L.S.	− 35	,,
	31	,,	,,	,,	0·02	,,	,,	S.S.	+ 15	,,
	32	,,	,,	,,	> 0·02	,,	,,	*nil*	+ 30	,,
	33	0·5	0·042	83	0·05	,,	,,	S.S.	0	,,
	34	,,	,,	,,	0·04	,,	,,	S.S.	+ 10	,,
	35	,,	,,	,,	0·3	,,	,,	*nil*	+ 25	,,
	36	,,	,,	,,	0·3	,,	,,	*nil*	+ 20	,,
	37	,,	,,	,,	0·02	,,	,,	V.L.S.	− 20	,,
	38	,,	,,	,,	0·01	,,	,,	V.L.S.	− 30	,,
Toxin-antitoxin Mixture B.671	39	1·0	2·24	75	0·005	83	0·02	L.S.	− 15	† 4½ days
	40	,,	,,	,,	0·01	88	,,	V.L.S.	− 40	† 7½ ,,
	41	,,	,,	83	0·5	,,	,,	*nil*	+ 20	Lived
	42	,,	,,	,,	0·05	,,	,,	V.S.S.	0	,,
	43	,,	,,	,,	0·005	,,	,,	—	− 25	† 1½ days
	44	,,	,,	,,	0·01	,,	,,	L.S.	− 30	Lived
	45	,,	,,	,,	0·2	,,	,,	*nil*	+ 5	,,
	46	,,	,,	,,	0·04	,,	,,	*nil*	+ 10	,,

† = died. V.S.S. = very small swelling. L.S. = large swelling. S.S. = small swelling. V.L.S. = very large swelling. M.S. = medium swelling. *nil* = no local reaction. — = died before the end of the 4th day.

The weight changes recorded are those which occurred at the end of 4 days after inoculation of the toxin, except in the case of animals which died in less than 4 days.

two-hundredth of a unit in two cases to a fifth of a unit in one case and half a unit in another. Five animals survived the injection of 3 M.L.D. of toxin and 3 died.

In the series of 10 animals injected with the precipitate from Mixture 7 antitoxin production was higher than in the other three series. Only 1 animal failed to produce a hundredth of a unit; 2 produced a hundredth, 3 a fiftieth, 1 a twenty-fifth, 1 a twentieth and 2 as much as three-tenths of a unit. All 10 animals survived the injection of 3 M.L.D. of toxin.

Forty-two of the 46 animals used in this experiment were injected with the test-dose of toxin on the same day, and the effect of the injection was observed daily. The increased resistance of toxin manifested by the series which had been immunized by injection of the precipitate from Mixture 7 was very evident. With one exception they remained well, fed normally and appeared to be unaffected by the toxin injection; the remaining animal ate little for a few days, but ultimately recovered.

In contrast with the animals of this series, 12 of the 14 animals which had received injections of the precipitate from Mixture 5 were obviously very ill on the day following the injection and 11 died within 48 hours. In the other two series some animals were apparently unaffected by the toxin, others died, while a number fed but little for a few days, but ultimately recovered. The same order of variation was observed with regard to the local reaction and the change in weight following the injection of the test-dose of toxin.

It may be pointed out that in the whole series of animals used in Experiment 4, of the 19 whose serum contained less than a hundredth of a unit of antitoxin 17 died after the injection of the test-dose of toxin; and of the 9 which produced a hundredth of a unit of antitoxin 7 survived and 2 died, one in $2\frac{1}{2}$ days and one in $7\frac{1}{2}$ days. The remaining 28 animals produced antitoxin in amounts varying from a fiftieth to half a unit and all survived the test-dose of toxin.

The antigenic activity of the precipitate from Mixture 7 is brought out in an even more striking way when the figures for the nitrogen content of these different preparations are considered. It has been pointed out that this precipitate formed slowly and was small in amount. The total nitrogen of the whole of the precipitate obtained from this mixture amounted to 0·84 mgm., and the total nitrogen of the whole original mixture was 468 mgm. That is to say, 99·8 per cent. of the nitrogen of the original toxin-antitoxin mixture has been removed, and there remains this small residue containing little more than traces of nitrogen, and yet capable, in very small doses, of evoking a very considerable immunity response in inoculated animals; a quantity of the precipitate, containing less than a hundredth of a milligramme of nitrogen, proved to be as effective an antigen as 1 c.cm. of an ordinary toxin-antitoxin mixture which contained 2·24 mgm. of nitrogen, or more than 250 times as much.

Further, according to the recent experiments of Flössner and Kutscher (1924), it appears that only a small portion of the nitrogen of these precipitates is in organic combination. They obtained 0·9683 grm. of precipitate from a mixture consisting of 6500 c.cm. of toxin and 180 c.cm. of antitoxin,

and analysis showed that 3·65 per cent. of this was soluble in alcohol and ether ; two-thirds of this fraction consists of cholesterol and the remainder of lipoid. 96·35 per cent. was insoluble in alcohol and ether, and of this an amount equal to 10 per cent. of the total precipitate is "protein"; the remainder—86 per cent.—consists of ammonium magnesium phosphate. Even on the improbable assumption that the nitrogen in organic combination in these precipitates is "antigen" and nothing else, these experiments show that an amount of antigen containing a thousandth of a milligramme of nitrogen suffices to immunize guinea-pigs. Four such animals (Nos. 29 to 32) developed antitoxin in their blood, and all survived the injection of 3 M.L.D. of toxin. This preparation was not tested at lower doses.

While this paper was being prepared for the press, two references to work on the same subject have appeared. Park, Banzhaf, Zingher and Schroder (Dec., 1924), in a table containing the results of the immunizing value of some toxoid and toxin-antitoxin preparations, quote figures showing the immunizing response to "T.A. solution of precipitate from mixing toxin and antitoxin." Two preparations were studied : of the first, 1 c.cm. killed guinea-pigs in 30 days, and 12 out of 15 guinea-pigs which received 0·2 c.cm. doses gave a negative Schick reaction at the second test carried out 4 weeks after the primary stimulus. The second preparation caused slight paralysis in doses of 1 c.cm., and the whole of 15 guinea-pigs, tested in the same way as those of the other series, were immunized by injection of 1 c.cm. quantities. No details of the preparation are given, and no comment is made in the text regarding these results. If it can be assumed that these experiments were carried out in a similar way to those described in the foregoing pages, it would appear that my results are in agreement with those of Park and his colleagues. Sordelli and Serpa (March, 1925) prepared precipitates from over-neutralized mixtures and centrifuged the flocculated mixture. They do not appear to have washed the precipitate. They found that the supernatant fluid produced a greater local reaction than the precipitate, and that the immunizing value of the two was about the same (*cf.* Experiments 2 and 3 above). They immunized rabbits and guinea-pigs by intracutaneous injection of the precipitate, and also by scarification. They state that a solid active immunity followed two scarifications and application of precipitate, and consider that the material, and the scarification method, can be employed for the active immunization of man.

CONCLUSIONS.

(1) The precipitates produced from mixtures of diphtheria toxin and antitoxin are insoluble in normal saline, and when they have been repeatedly washed with this fluid contain less than 1 per cent. of the nitrogen compounds of the original mixture.

(2) All the preparations of this kind studied in this series of experiments were innocuous to guinea-pigs, even in large doses. With one exception, they were all prepared from mixtures in which the antitoxin was in excess.

(3) Such washed precipitates are antigenic. Guinea-pigs injected with amounts of precipitate-emulsion containing less than a hundredth of a milligramme of nitrogen (and less than a thousandth of a milligramme of organic

nitrogen) became immune to diphtheria toxin. They developed antitoxin in their blood and survived the injection of three fatal doses of diphtheria toxin.

(4) The antigenic value of the precipitate varies with the nature of the original toxin-antitoxin mixture. If antitoxin is in very large excess the antigenic value of the resulting precipitate is low. The antigenic value of precipitates prepared from mixtures containing a moderate excess of antitoxin is, however, considerable. The most active preparation was obtained from a mixture which was slightly toxic for guinea-pigs.

REFERENCES.

Berthelot, A., and Ramon, G.—(1925) "Sur les agents de transformation des toxines en anatoxines," *C.r. Acad. d. Sci.*, **180**, 341.

Flössner, O., and Kutscher, Fr.—(1924) "Zur Kenntnis der Ramonschen Flockungsreaction," *Münch. med. Wchnschr.*, **71**, 576.

Glenny, A. T., and Südmersen, H. J.—(1921) "Notes on the Production of Immunity to Diphtheria Toxin," *J. Hyg.*, **20**, 176.

Glenny, A. T., Allen, K., and Hopkins, B. E.—(1923) "Testing the Antigenic Value of Diphtheria Toxin-antitoxin Mixtures," *Brit. J. Exper. Pathol.*, **4**, 19.

Glenny, A. T., and Hopkins, B. E.—(1923) "Diphtheria Toxoid as an Immunising Agent," *ibid.*, **4**, 283.

Glenny, A. T., and Okell, C. C.—(1924) "The Titration of Diphtheria Toxin and Antitoxin by Flocculation Methods," *J. Path. & Bact.*, **27**, 187.

Park, W. H.—(1922) "Toxin-antitoxin Immunization against Diphtheria," *J. Amer. Med. Assoc.*, **79**, 1584.

Park, W. H., Banzhaf, E. J., Zingher, A., and Schroder, M. C.—(1924) "Observations on Diphtheria Toxoid as an Immunizing Agent," *Amer. J. Public Health*, **14**, 1047.

Ramon, G.—(1922[1]) "Floculation dans un mélange neutre de toxine-antitoxine diphtériques," *C.r. Soc. de biol.*, **86**, 661.—(1922[2]) "Sur une technique de titrage in vitro du sérum antidiphtérique," *ibid.*, **86**, 711.—(1922[3]) "A propos du titrage in vitro du sérum antidiphtérique par la floculation," *ibid.*, **86**, 813.—(1923[1]) "Sur la concentration du sérum antidiphtérique et l'isolement de l'antitoxine," *ibid.*, **88**, 167.—(1923[2]) "Dissociation du complexe toxine-antitoxine diphtérique et récupération d'antitoxine," *C.r. Acad. d. Sci.*, **176**, 267.—(1923[3]) "Pouvoir floculant et pouvoir toxique de la toxine diphtérique," *C.r. Soc. de biol.*, **89**, 2.—(1923[4]) "Sur le pouvoir floculant et sur les propriétés immunisantes d'une toxine diphtérique rendue anatoxique (anatoxine)," *C.r. Acad. d. Sci.*, **177**, 1338.—(1923[5]) "La floculation dans les mélanges de toxine et sérum antidiphtérique," *Ann. de l'Inst. Pasteur*, **37**, 1001.—(1924[1]) "Des anatoxines," *C.r. Acad. de Sci.*, **178**, 1436.—(1924[2]) "Sur les propriétés de l'anatoxine diphtérique," *ibid.*, **179**, 422.—(1924[3]) "Sur l'apparition des anticorps," *ibid.*, **179**, 485.—(1924[4]) "Sur la toxine et sur l'anatoxine diphtérique. Pouvoir floculant et propriétés immunisantes," *Ann. de l'Inst. Pasteur*, **38**, 1.—(1925) "Sur l'anatoxine diphtérique et sur les anatoxines en général," *ibid.*, **39**, 1.

Schroder, M. C., and Park, W. H.—(1923) "Comparative Merits of New and Old Preparations of Diphtheria Toxin-antitoxin. Results of Preliminary Tests with Toxoid," *J. Amer. Med. Assoc.*, **81**, 1432.

Sordelli, A., and Serpa, R.—(1925) "Vaccination antidiphtérique," *C.r. Soc. de biol.*, **92**, 824.

Zingher, A., and Park, W. H.—(1924) "Immunity Results obtained in School Children with Diphtheria Toxoid (Modified Toxin), and with 1/10 L_+ Mixtures of Toxin-antitoxin," *Proc. Soc. Exper. Biol. & Med.*, **21**, 383.

THE VIRUS OF ENCEPHALITIS LETHARGICA.

J. R. PERDRAU.

From the Laboratory of the Lambeth Hospital, London.

Received for publication April 23rd, 1925.

RESULTS OF SIMPLE INTRACEREBRAL INOCULATIONS INTO LABORATORY ANIMALS.

In a recent communication (Perdrau, 1925), the results of intracerebral inoculations into rabbits of brain emulsions from fatal cases of encephalitis lethargica have been recorded in some detail.

Although in no instance could a transmissible virus be isolated, a peculiar syndrome, characterized by lethargy, was obtained in about half of the animals inoculated. The brains of these animals showed in many cases some mild inflammatory reactions.

It was pointed out at the same time that these results were identical with those obtained in animals, immunized with the virus of herpes, which finally succumb to an intracerebral inoculation of that virus owing to the disappearance of their immune state after a certain lapse of time.

It is clear, therefore, that a simple cerebral inoculation into rabbits is not the method of choice for the recovery of the virus from the brain of fatal cases of encephalitis lethargica.

Material other than brain was also used, and a brief account of the results obtained are given here.

Cerebrospinal fluid from 3 cases of encephalitis lethargica was injected intracerebrally 5 times with 2 deaths, but no evidence of an encephalitis was obtained histologically.

Corneal inoculations with the brain of 8 cases were carried out 11 times, and a mild keratitis without any conjunctivitis was obtained twice, both from the same case. All attempts at further passages, either corneally or cerebrally, failed completely.

Some brain from a fatal case of the disease received by Dr. Da Fano from Professor Kling of Stockholm and placed at my disposal by the former was injected into 6 rabbits, 5 of which died within one month. The symptoms and histological lesions in the brain were similar to those obtained with material derived from cases occurring in this country. Further passages were tried without success.

The unfiltered nasal washings of 7 cases of encephalitis lethargica were inoculated on the cornea of 15 rabbits, and a simple keratitis without any conjunctivitis was obtained on 11 occasions. Six out of 7 washings proved infective. In one case the rabbit developed a state of active immunity to the virus of herpes for a period of one month. Dr. P. P. Laidlaw (private communication) filtered a number of nasal washings and injected them intrathecally into rabbits with negative results. A few attempts were made to

infect guinea-pigs and mice without any success, whilst Dr. P. P. Laidlaw kindly inoculated 3 monkeys (*Macacus sinicus*) by the cerebral route with the brain of 3 fatal cases of the disease and they all survived.

The presence in the nose of 6 out of 7 unselected cases of encephalitis lethargica of a virus, capable of producing a keratitis, is certainly suggestive of a close relationship between that virus and the disease from which the patient suffered, but the proportion of the numbers of healthy subjects who are carriers of such a virus is still unknown. For the time being one must classify the virus present in these nasal washings and in one of the brains examined and found capable of giving rise to a keratitis with that of herpes.

In all the animals which died in the course of these experiments, the spontaneous encephalitis of rabbits was excluded by histological examinations.

The results obtained by simple intracerebral inoculations of emulsions of the brain of fatal cases of encephalitis lethargica into rabbits were therefore of the same negative character as those obtained by other investigators.

PRESENCE OF AN AGGRESSIN-LIKE FACTOR UNDER CERTAIN CONDITIONS OF EXPERIMENT.

The experiments recorded so far were carried out in 1923 and 1924. In the meanwhile as the result of a study of the immunity reactions of the virus of herpes, the conclusion was arrived at that the viruses of herpes and of encephalitis lethargica are probably identical. This view has been upheld chiefly by Levaditi and his associates. As the result of the experience gained with the virus of herpes a fresh series of experiments was undertaken during the recent winter months, but owing to the comparatively low incidence of the disease material was obtained from only 3 fatal cases. The following are some of the reasons which appeared to justify a fresh attempt at the recovery of the virus of the disease. The theory was put forward that in cases of encephalitis lethargica when the virus reaches the central nervous system one of three things happens, viz. (1) development of a local cellular immunity which overcomes the infective agent and leads to a complete recovery; (2) the development of a state of cellular immunity which only partially overcomes the infection, the final issue being either a fatal one or a chronic infection; (3) failure of development of any immunity and a quickly fatal result. The last category would account for those few cases from whose brain or cerebrospinal fluid a virus identical with that of herpes had been isolated by direct cerebral inoculation into susceptible animals. But it is the second class which provides the vast majority of fatal cases, because direct cerebral inoculations of emulsions of brain into susceptible animals have failed so far to lead to the recovery of a virus transmissible in series. On the other hand, in the same paper it had been shown that under certain conditions of experiment there were indications of the presence of an activating substance, possibly of the nature of an aggressin, in the brain of rabbits inoculated with the virus of herpes. For instance the incubation period was considerably reduced if the animal received two intracerebral inoculations, or a cutaneous one followed by a cerebral at 48 hours' interval. Again, the addition of emulsions of the brain of animals immunized by cutaneous inoculations of the

passage virus markedly shortens the incubation period for intracerebral inoculations. The presence of the activating substance could be demonstrated more easily in the last case if the immune brain was kept in glycerin in the ice-chest for about a fortnight.

These observations were the guiding factor in the following series of experiments.

DESCRIPTION OF NEW TECHNIQUE.

Pieces of the mid-brain and of the basal ganglia from 3 acute cases of encephalitis lethargica were received preserved in glycerin. In none of these cases were any herpetic lesions noticed during life. Case 1 died 8 days, Case 2, 60 hours, and Case 3, 4 days after the onset of symptoms.

Simple cerebral inoculations of emulsions of these brains into rabbits remained without result, when the material had been only 2 to 3 days in glycerin. Attempts were next made to increase the virulence of the virus either by adding to the inoculum the activating substance referred to already, or by the production of the latter in the animal experimented upon. In the first case the activating substance added to the emulsions of human brain consisted of an emulsion in normal saline of the brain of a rabbit which had received two sets of intradermal inoculations of the passage virus of herpes on 2 successive days, and which was killed 48 hours after the second inoculation. The brain of the rabbit was used after it had stayed at least 2 and not more than 3 weeks in glycerin in the ice-chest. The brain of an immunized animal acts just as well, if not better, under similar conditions of preservation, but there is a great saving of time by using the former. In the second case, that is to say for the production of the activating substance (henceforward referred to as an "aggressin," although its exact nature must remain in doubt for the time being) in the animal under experiment, emulsions of the human brain to be tested were injected repeatedly, intradermally and intracerebrally, usually on alternate days, for 4 consecutive days, with or without the addition of the "aggressin" in the case of the cerebral inoculations.

It should be stated that the inoculation of the "aggressin" brain into rabbits by the various methods given here failed to show the presence of the virus itself in any of them, although an intradermal inoculation into a new rabbit usually leads to a little local redness. The same negative result attended the histological examinations.

RESULTS OBTAINED WITH NEW TECHNIQUE.

The results obtained with Case 1, being the most illustrative of the three, will be given in full, as considerable variations in virulence were noticed according to the length of time that the human brain had been kept in glycerin in the ice-chest.

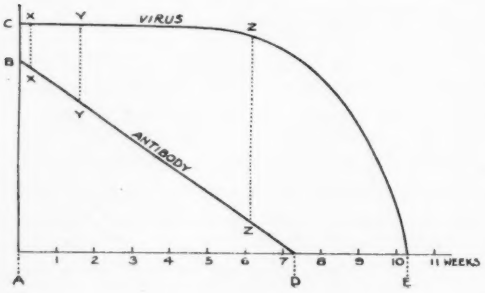
CASE 1.—After 2 days in glycerin a single intracerebral inoculation remained without result. But when an intradermal inoculation was followed on the next day by an intracerebral inoculation, and on each of the next 7 days by cutaneous inoculations given by deep scarification, the scrapings of each scarification being used for the next, the rabbit developed the typical

herpetic syndrome on the 12th day and died on the 14th day. The usual histological picture of a herpetic encephalitis was present in its brain and the virus was easily passaged. In order to determine whether the "aggressin" which had been added to the inoculum for the cerebral inoculation was responsible for the positive result, three rabbits were inoculated as follows with emulsions of the brain of the same case after it had been 17 days in glycerin: R 431 received a single cerebral inoculation of the human brain alone, R 432 received on 3 consecutive days an intradermal, a cerebral and an intradermal inoculation of human brain alone, respectively, and R 433 received a single intracerebral inoculation of a mixture of human brain and "aggressin." R 431 made a complete recovery and incidentally was found to have been immunized by the inoculation; R 432 and R 433 died in 6½ days with a typical herpetic syndrome. (Virus E.L. 1 is derived from R 432, in which no "aggressin" was used.)

This experiment clearly shows that the "aggressin" cannot be the virus itself, especially in view of the fact that repeated inoculations of a concentrated emulsion intradermally and intracerebrally into new rabbits remain without effect.

A point of great interest in the experiments just recorded is the reduction of the incubation period from 14 to 7 days, although the material had been longer in glycerin. When an emulsion prepared from the brain of the same case, after it had been in glycerin for 43 days, was injected intracerebrally into a new rabbit, the latter developed the typical herpetic syndrome and died within 4 days. It is clear, therefore, that there must have been a great increase in the virulence of the virus during the 15 days that had elapsed since the last experiment. The emulsions used were prepared from small pieces taken from the same areas in every instance. The possibility of the virus having grown in the preserving fluid in the ice-chest is excluded by a large series of experiments in which liquid culture media containing glycerin were used. In every instance the virus was found to die rapidly in them and never to diffuse into the medium. The most likely explanation seems to be that according to the theory of the production of an antibody in infected individuals, the rate of degradation of the virus and of the immune substance, formed in response to the former, are not coincident. The accompanying diagram seems to be a more correct explanation of what actually takes place.

This diagram tries to show what seems to have taken place from the moment that the virulent brain was placed in glycerin in the ice-chest. AC represents the amount of virus present, and AB the amount of antibody which has neutralized the greater part, but not the whole, of the virus, so that there is still a little free virus left. The rate of degradation of the antibody (B–D) is gradual and steady, but that of the virus



(C–E) is slow at first and more rapid later. At X (2 days) the small amount of unbalanced virus produced no effect by itself, but with the help of the activating substance it killed a rabbit in 14 days. At Y (17 days)* the amount of free virus is larger and is sufficient to immunize an animal, and with the help of the "aggressin" it kills in 6½ days. At Z (43 days) the amount of unbalanced virus is still larger and kills by itself in 4 days.

Should this explanation be the correct one, the ideal to be sought for is obviously to find out some means of destroying the immune substance, or of separating it from the virus. Lack of material from fatal cases of encephalitis lethargica has so far impeded this work. It is interesting to recall here that Levaditi (1922) had suspected the presence of an antibody ("substance empêchante") in fresh virulent brain. A similar observation, whose significance was not fully appreciated at the time, has often been made in the course of this research, namely, that the passage virus is more virulent after a sojourn of a few days in glycerin in the ice-chest than when used fresh. If the theory already referred to is correct, it is clear that the amount of unbalanced virus (B–C) in the brains of fatal cases of encephalitis lethargica will vary considerably in each individual case. This is borne out by a study of two other fatal cases of the disease. In Case 1 the amount of free virus must have been comparatively large, in Case 2 it was much less, and in Case 3 still less.

Case 2 died 60 hours after the onset of acute symptoms. Intracerebral inoculation of an emulsion of the brain of this case with the addition of some herpetic "aggressin" as used for Case 1 led to no symptoms when the brain had been 4 days in glycerin. After 34 days in glycerin, intradermal and intracerebral inoculations given alternately on 4 consecutive days led to the production of the typical herpetic syndrome in 7 days. The histological examination showed an encephalitis of the usual type. Unfortunately owing to a shortage in the supply of animals the virus was not passaged immediately, and when it could be done the virus was found to be dead. At no time did a simple intracerebral inoculation of emulsions of the brain of this case produce any symptoms when used by itself. The amount of unbalanced virus must have been appreciably less than in Case 1.

In Case 3, who died 4 days after the onset of acute symptoms, but who had probably been suffering from a mild infection for 5 months previously, an attempt was made to increase the virulence of the virus by intradermal passages. The first inoculation led to very little local reaction, but the next two passages showed a very obvious one. At the third passage the presence of the virus was demonstrated by its ability to produce a very definite keratitis when injected by the corneal route. In this case the technique employed in Case 2 failed to give any results.

It has therefore been possible by the adoption of a special technique to demonstrate the presence of a virus, indistinguishable from that of herpes, in the brain of all three cases of encephalitis lethargica in which this technique has been employed. From one of the cases (Case 1) a fixed virus has been obtained, which has been shown to be identical with that of herpes, including cross-immunity experiments. The only difference noted so far is a slightly

* Shown incorrectly in the diagram as 11 days.

greater virulence as shown by a shorter incubation period (4 days) in the case of the encephalitic virus (virus E.L. 1).

CONCLUSIONS.

Simple intracerebral inoculation of cerebrospinal fluid, and of emulsions of brain from cases of encephalitis lethargica into rabbits and other laboratory animals, is not the method of choice for the recovery of the virus of the disease.

A technique is described by which the presence of a virus indistinguishable from that of herpes was demonstrated in the brains of all 3 of the cases in which this technique has been used.

This technique is based on the presence of an activating substance, possibly of the nature of an aggressin, in the brain of animals under certain conditions of experiment.

A virus capable of giving rise to a keratitis in the rabbit, and apparently identical with that of herpes, was demonstrated in the nasal washings of 6 out of 7 cases of the disease.

The investigations undertaken in the spring of 1924 were carried out under the best conditions one could wish for as regards the collection of material, and were only rendered possible through the active co-operation of Prof. A. Hall and Prof. J. S. C. Douglas, of Sheffield University, to whom I wish to record my gratitude. My thanks are also due to Dr. Sutherland, of the Monsall Hospital, Manchester; Dr. Rundle, of the City Hospital, Liverpool; Dr. Archibald, Belvidere Hospital, Glasgow, and many others for specimens from cases of encephalitis lethargica.

To Dr. P. P. Laidlaw I am indebted not only for much valuable advice, but also for his help in kindly undertaking many investigations, and for placing his results at my disposal.

This research has been carried out with the help of a grant from the Medical Research Council.

REFERENCES.

LEVADITI, C.—(1922) 'Ectodermoses Neurotropes.' Paris. (Masson & Cie.) p. 168.
PERDRAU, J. R.—(1925) *Brit. J. Exper. Pathol.*, **6**, 41.

THE ÆTIOLOGY OF MILK FEVER IN CATTLE.

WILLIAM LANCELOT LITTLE AND NORMAN CHARLES WRIGHT.

From the National Institute for Research in Dairying, University College, Reading.

Received for publication May 6th, 1925.

THE literature relating to the ætiology of milk fever in cattle is extensive: for a detailed account reference may be made to any standard text-book on veterinary science. It will suffice for us to note here that the symptoms of milk fever have been attributed to such diverse conditions as cerebral anæmia, cerebral hyperæmia, the formation of fat emboli, auto-intoxication, anaphylactic shock, and mammary neurasthenia. In no case, however, is there direct experimental evidence which supports any one of these theories.

The object of this paper is to record the results obtained in an investigation which has been carried out at this Institute since November, 1924. The number of determinations is small, but the very consistent results obtained, together with the difficulty in securing further cases for investigation, have led us to issue this preliminary report.

The investigation had its origin in the observation that the tetany of milk fever presented very similar clinical symptoms to those which have been found by Macallum and Voegtlin (1909) and other workers to be associated with the lowered calcium content of the blood of parathyroidectomized animals. The loss of calcium during the later period of pregnancy is considerable, and it was suggested that at parturition conditions might exist in which the blood calcium content could be more easily lowered. Our work commenced, therefore, with estimations of the blood calcium content of cows affected with milk fever, and after recovery.

During the progress of the work two papers have appeared in the *Veterinary Record*, in which further theories of the ætiology of milk fever have been brought forward. The first is that of Pugh (1924), in which he advances the theory that milk fever is caused by botulinus-like toxins, following the onset of ketosis due to faulty fat metabolism. The second is that of Dryerre and Greig (1925), in which the symptoms are put down to guanidine intoxication resulting from a relative parathyroid deficiency. No experimental evidence is offered in support of either of these theories.

In order to verify the statements made by Pugh, we have included, during the latter period of our investigation, the quantitative estimation of acetone bodies in the blood and urine of cows with milk fever. The results of these determinations are noted in the second part of this paper.

PART I.—DETERMINATION OF BLOOD CALCIUM CONTENT.

*Method.**—Determinations were carried out on samples of citrated plasma from blood taken from the jugular vein. The following technique was adopted:

* This method has now been used for more than one hundred determinations, with satisfactory results.

Duplicate samples of from 10 to 25 c.c. of plasma were taken for analysis, so that reasonable titration figures with N/100 $KMnO_4$ might finally be obtained. Each sample was ashed in platinum, and the ash digested with hydrochloric acid for at least one hour on a water-bath.

The solution was transferred to a 60 c.c. round-bottomed centrifuge vessel, and the calcium precipitated as oxalate by McCrudden's (1909) method: methyl red was substituted for alizarin red as indicator (Clark, 1921). The precipitate was centrifuged at 3000 r.p.m., the supernatant liquid siphoned off, and the precipitate washed twice with 25 c.c. distilled water, being centrifuged between each washing. The precipitate was finally dissolved in normal H_2SO_4, heated to 75°C., and titrated with N/100 $KMnO_4$.

Results.—The results are set out in Tables I–IV and Figs. 1 and 2.

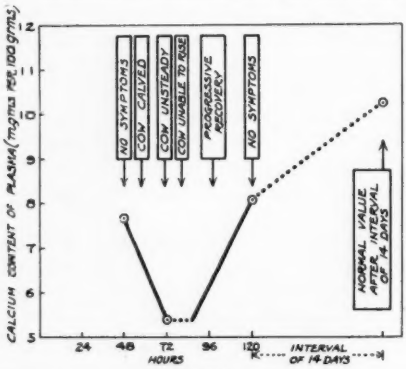


Fig. 1.

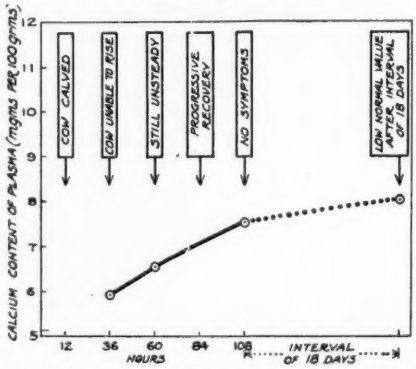


Fig. 2.

Fig 1.—Illustrating the alterations in calcium content of the blood-plasma of Case 4. Note the fall in calcium *before* calving, and the relatively quick recovery to normal.

Fig. 2.—Illustrating the very slow rise in the calcium content of the blood-plasma of Case 3. Note also the low normal value.

Table I.—*Normal Values of Blood Calcium Concentration.*

	Calcium content of plasma (mgm. per 100 gm.)	
	Duplicates.	Mean.
Case 13	{ 9·70 / 10·10 }	9·9
„ 14	{ 9·60 / 10·00 }	9·8
„ 15	{ 10·40 / 10·00 }	10·20
„ 16	{ 10·20 / 9·80 }	10·00
„ 17	{ — / 9·80 }	9·80
„ 18	{ — / 10·40 }	10·40

TABLE II.—*Blood Calcium Concentration over a period of 10 weeks.*

Variation of calcium content of plasma (mgm. per 100 gm.).	Number of determinations.
Above 10·5	0
9·0 to 10·5	11
8·0 to 9·0	2 (after administration of salts)
7·0 to 8·0	1 („ „ „)
Below 7·0	0

TABLE III.—*Blood Calcium Content.*

Case No.	Cow "down." Duplicates	Cow "down." Mean.	Cow normal. Duplicates.	Cow normal. Mean.	Remarks.
	(Mgm. of Ca per 100 gm. of plasma)				
1	4·50, —	4·50	9·50, 9·70	9·60	Typical case.
2	4·15, 4·50	4·35	9·40, 9·30	9·35	„ „
4	5·60, 5·10	5·35*	10·50, 10·30	10·40	* Sample taken just before onset of milk fever. Typical case.
6	6·90, 7·30	7·10	—, —	—	Mild symptoms. Cow sold before normal sample could be taken.
7	4·05, 4·35	4·20	—, —	—	Typical case. Cow sold before normal sample could be taken.
8	4·10, 4·05	4·10	10·30, 10·60	10·45	Very severe case. Cow unconscious.
11	5·10, 5·25	5·20	8·25, 8·15	8·10	Typical case. Normal sample taken 24 hours after recovery.
12	6·30, 6·35	6·35	9·50, 10·00	9·75	Typical case.

Table I gives the values obtained on 6 normal cows. These are in agreement with the figures obtained by other workers. Meigs (1922) gives an extensive bibliography, in which he notes the normal values as varying from 9 to 11 mgm. per 100 c.c. plasma.

Table II shows the average variation in the blood calcium concentration of 1 cow over a period of several weeks. During part of this time the cow was used experimentally in an investigation on the influence of oral ingestion of inorganic salts on the composition of milk. The details of these experiments will be published elsewhere, but the figures recorded are sufficient to show the relatively small normal variation over an extended period.

Table III gives the results obtained from 8 cows "down" with milk fever, and the normal values after recovery. In all these cases the recovery to normal was rapid; a typical curve illustrating this rapid rise is shown in Fig. 1.

Table IV gives the results obtained from 4 cows "down" with milk fever. All these cases were taken from the same farm, and the striking point illustrated in the table is the low *normal* value of the blood calcium concentration. Coinciding with this low normal value should be noted the very slow recovery which is illustrated in Fig. 2. The results obtained on this farm indicate that dietetic factors may play an important part in the causation of milk fever; further work on the subject is, however, being continued at this Institute.

TABLE IV.—*Blood Calcium Content.*

Case No.	Cow "down." Duplicates.	Cow "down." Mean.	Cow normal. Duplicates.	Cow normal. Mean.	Remarks.
	(Mgm. of Ca per 100 gm. of plasma.)				
3	6·00 5·70	5·85	7·95 7·90	7·95	Mild symptoms. Normal sample taken 3 weeks after recovery.
5	6·40 6·10	6·25	7·80 7·80	7·80	Mild symptoms. Normal sample taken 2 days after recovery.
9	3·60 3·80	3·70	6·70 6·95	6·85	Normal sample taken 4 days after recovery. Typical case.
10	5·75 5·95	5·85	7·75 7·85	7·80	Typical case. Normal sample taken 24 hours after recovery.

DISCUSSION.

It is at once apparent that the onset of milk fever is accompanied by a greatly reduced concentration of calcium in the plasma, the severity of the case being paralleled by a proportionately large decrease in the calcium

concentration; thus with mild symptoms reductions of 20 to 30 per cent. were obtained, while with severe symptoms the fall was as much as 60 per cent. of the normal. In an interesting paper Dryerre and Greig have suggested that milk fever is primarily due to a relative parathyroid deficiency, leading to guanidine intoxication of the tissues. In such a case of parathyroid deficiency we should expect the blood calcium content to be lowered, so that to this extent our work tends to support their hypothesis. At the same time it should be pointed out that parathyroid deficiency is not an invariable accompaniment of tetany. Tetany can be induced by various means: by dosage with large quantities of bicarbonate, by voluntary over-breathing, by injection of phosphates into the blood-stream, or as a result of gastric obstruction. There is no evidence that the tetany produced by such means is primarily the result of guanidine intoxication; it would appear to be due rather to faulty salt balance in the blood or to reduction in the ionized calcium.

We are, in fact, still unable to say whether the tetany resulting from parathyroid deficiency itself is due to guanidine intoxication or to calcium deficiency, or to some other factor of which these two are accompaniments. The same difficulty confronts us with regard to milk fever.

While the problem can only be solved when we have a sufficient mass of experimental data at our disposal, we feel that the evidence in this paper, which, we believe, constitutes the first definite experimental evidence put forward in connection with the disease, is of sufficient importance to warrant immediate publication.

PART II.—DETERMINATION OF ACETONE BODIES.

Method.—The method used was that of Van Slyke and Fitz (1917), in which the "total acetone bodies" are estimated by precipitation as mercury-chromate-sulphate compound after removal of interfering substances. By this method the aceto-acetic acid and β-hydroxy-butyric acid present are first oxidized with bichromate: the "total acetone bodies" therefore represent both the acetone present as such, and also that formed from the bichromate oxidation.

Results.—The results are recorded in Tables V and VI:

TABLE V.—*Acetone Bodies in Blood.**

Cow.	Mgm. per litre.	Remarks.
Brenda	15	Normal
Case 5	10	,,
,, 6	8	Mild case
,, 5	16	,, ,,
,, 7	15	Severe case
,, 9	10	,, ,,

TABLE VI.—*Acetone Bodies in Urine.*

Cow.	Gm. per litre.	Remarks.
Doll	0·42	Normal
Carlet	0·14	,,
Bertha	0·18	,,
Doll (2nd sample)	0·08	,,
Case 8	0·77	Severe case
,, 9	0·13	,, ,,
,, 10	0·10	Mild case

* It should be noted that citrated plasma cannot be used for this determination, owing to the formation of acetone by oxidation of citric acid.

Discussion.

Van Slyke and Fitz, using this method, have shown that the normal variation in the blood is from 0·013 to 0·026 gm. per litre, and in the urine from 0·08 to 0·30 gm. per litre. In acute pathological conditions (for instance, in diabetic coma) the values may rise to as much as 2·5 gm. per litre for blood, and 10 to 12 gm. per litre for urine. Pugh states that in milk fever "quantitative analysis has shown aceto-acetic acid is present in far greater amounts that can be explained on physiological grounds." We should be interested to see his figures, since our own indicate the reverse of this conclusion.

SUMMARY.

Determinations of the calcium content of the blood-plasma of cows suffering from milk fever show that considerable diminutions (in mild cases 20 to 30 per cent., in severe cases up to 60 per cent.) accompany the onset of this disease.

We have been unable to demonstrate any significant increase in the "total acetone bodies" of the blood or urine in these cases.

REFERENCES.

Clark, G. W.—(1921) *J. Biol. Chem.*, **49**, 487.
Dryerre, H., and Greig, J. R.—(1925) *Vet. Record*, **5**, 225.
Macallum, A. B., and Voegtlin, C.—(1909) *J. Exper. Med.*, **11**, 118.
McCrudden, F. H.—(1909) *J. Biol. Chem.*, **7**, 83; **10**, 187.
Meigs, E. B.—(1922) *Physiol. Reviews*, **2**, 228.
Pugh, L. P.—(1924) *Vet. Record*, **9**, 999.
Van Slyke, D. D., and Fitz, R.—(1917) *J. Biol. Chem.*, **32**, 455.

STUDIES ON ŒDEMA: I. THE ELECTROLYTE CONCENTRATION IN THE BODY FLUIDS IN NEPHRITIS WITH ŒDEMA.

JOHN MARRACK.

From the Hale Clinical Laboratory, London Hospital.

Received for publication May 28th, 1925.

THE idea that a high concentration of electrolytes is found in the body fluids of patients with nephritis and œdema dates largely from the time when chloride estimations were made on whole blood, and high values found therein owing to the deficiency of red blood-corpuscles, with their relatively low chloride content; also from the time when it was not understood that the higher chloride concentration in œdema fluids was simply the consequence of their low protein content. However, Denis and Hobson (1923) have found great excess of sodium in the serum in some cases of nephritis with œdema; and Fodor and Fischer (1922) finding high plasma chlorides, and still higher chlorides in the effusions of their patients, attribute œdema to excess of electrolytes in blood and tissue fluids.

It frequently has been observed that plasma chlorides are usually within normal limits in nephritis with œdema, and that low values are sometimes encountered; but it cannot be inferred from this alone that the total electrolytes are not increased, as salts of other acids may be present in excess; unless all the possible acids are estimated it is not possible to draw conclusions as to the total concentration of electrolytes from the amount of acid present. However there is no reason to suppose that any bases, other than the four mineral bases Na, K, Ca and Mg, are present in plasma in appreciable quantities. Since the acids present must be equivalent to the bases, it is possible to estimate the total electrolytes by determining the mineral bases separately or all together. The separate estimations would be satisfactory if it were not that there is some doubt as to the accuracy of the sodium method. It seems probable that too high values may be obtained from inadequate drying of the precipitate, or too low values from incomplete precipitation or loss of precipitate through the filter; it is certain that some of the values found by careful workers can hardly be considered correct.

The results obtained by separate estimations mainly indicate that there is no increase of electrolytes in œdema—Marrack (1923), Salvesen and Linder (1923), Rabinovitch (1924); but there remain the exceedingly high figures of Denis and Hobson (1923). It might be supposed that either the normal or the high figures were wrong.

For the analyses given in the table I have used a new method for estimating the total mineral bases of plasma. This is essentially the method used by Van Slyke, Wu and MacLean (1923), but the sulphate formed is estimated by the benzidin method as used by

Fiske (1921). This method has been used also by Cullen and Robertson (1924); the details of the method as they used it are, according to a private communication by Dr. Cullen, essentially the same as those worked out by Mr. G. D. Thacker and myself; since Cullen and Robinson propose to publish the method it is unnecessary to give the details here. The method is accurate to within 1 per cent.

In a few analyses serum was used, but, in the majority, plasma, which was obtained by using as an anticoagulant 3 mgm. of sulpharsenol, roughly weighed, to 20 c.c. of blood; a small correction was necessary for the sodium in the sulpharsenol. The blood was in all cases collected under oil in a centrifuge tube containing glass beads, the tube corked when full and shaken to dissolve the sulpharsenol or defibrinate. The sulpharsenol was not wholly satisfactory as an anticoagulant, as the plasma was liable to form, sooner or later, a gelatinous clot, which would not contract.

Bicarbonate was estimated by the titration method of Van Slyke (1922); chloride by a method similar to that published by Van Slyke (1923); water by the difference between the weight of a measured volume of plasma and the dry substance, obtained by evaporating on a water-bath and drying to constant weight in a desiccator.

Case.	Mgm. equivalents per litre.			Mgm. equivalents per 1000 gm. H_2O.			Grammes per litre.		Œdema.	Fluid used and remarks.
	HCO_3^-.	Cl^-.	Base.	HCO_3^-.	Cl^-.	Base.	Protein.	Blood urea.		
Normal (Min.)	—	—	150	—	—	159	—	—	—	—
Normal (Avge.)	28	103	155	29·8	109	164	7·7	—	—	Plasma.
Normal (Max.)	—	—	160	—	—	170	—	—	—	—
I.	23·5	108·6	145	—	—	—	40	0·20	+ +	Plasma. After 0·28 gm. NaCl per kilo. Plasma-water not estimated.
II. 26/2/25	26·5	99	143·5	27·8	104	150·6	47	0·32	+ +	Plasma.
5/4/25	18·8	104·5	142	19·4	109·5	149	47	—	+ +	Plasma.
5/3/25	23·5	111	142	23·7	112	143·4	Trace	—	—	Œdema fluid.
III.	28	105	151	29·5	110·5	159·4	31	0·32	+	Plasma.
	30·2	112	144·5	30·5	113	146	8	—	+	Ascitic fluid.
IV.	28·5	100	155	30·0	105·5	163·5	—	—	+	Œdema decreasing. Plasma.
V. 24/1/24	24	107·6	156·5	26·2	112·7	164	—	0·51	+	Œdema decreasing. Plasma.
13/2/24	25	106	156	26	110	162	—	—	+	„ stationary. Plasma.
VI.	26·5	103	151	27·8	108	158·5	42	0·87	+ +	Œdema decreasing. Plasma.
VII.	21·5	108·6	149·5	22·7	114·7	155·5	44	0·90	+ +	Plasma.
	22	122·5	152·5	22·2	123·7	154·0	Trace	—	+ +	Œdema fluid.
VIII.	15·5	107	146	16·5	113	155	47	1·00	+	Plasma.
IX.	26·5	96·5	149·5	29	102·8	159·5	55	118	+	Plasma.
	27	109	143	27·2	110	144·2	Trace	—	—	Ascitic fluid.

The concentration of total base in plasma varies slightly with the CO_2 tension and oxygenation of the blood owing to the migration of water in and out of the corpuscles (Van Slyke, Wu and MacLean, 1923). The normal range by this method is from 150 to 160 milligramme equivalents per litre, an estimate which agrees with those by other methods.

In the table I have also given the number of milligramme equivalents per 1000 grammes of water, as this is a more correct measure of the osmotic pressure than the number of milligramme equivalents per litre of solution.

In all the cases the concentration of base is below or within the lower limits of normal; even in Case 1, who after a dose of 0·28 grammes of NaCl per kilo had put on 0·3 kgm. in weight.

The chloride concentration is higher in the effusions than in the plasmata,

which is to be expected, as their protein content is much less; protein is an indiffusible anion, and in consequence the fluid with the lower protein content contains a higher concentration of diffusible anions, such as chloridion, according to Donnan's principle; for example, normal cerebrospinal fluid contains about 0·72 per cent. of chloridion, reckoned as sodium chloride, as compared with about 0·60 per cent. in plasma, which is equivalent to 0·65 grammes per 100 c.c. of plasma water.

In none of these cases therefore is there any evidence of abnormally high concentration of electrolytes in plasma or effusions. In the cases with high blood-urea, it might be supposed that water was retained to avoid an increase of osmotic pressure due to the retained urea, and that the low concentration of electrolytes was due to this water retention. However, the lowest concentration of electrolytes was found in cases in which there was no urea retention; and water retention sufficient to compensate for urea retention does not occur; thus a patient with a blood urea of 4·0 grammes per litre, that is an increase of over 60 millimols urea per litre, had a plasma total base of 147 milligramme equivalents per litre, that is a reduction of only 8 or less milligramme equivalents per litre. It seems probable that the osmotic pressure of urea inside the body is of no practical significance, as it is equally distributed through all the body fluids, its effect is as little felt as the atmospheric pressure under ordinary conditions.

In cases in which there is no retention of urea this low concentration of electrolytes should also manifest itself by a diminished lowering of freezing-point: this has been found by Gram (1923), and by Loeb, Atchley, and Palmer (1923). It has also an important bearing on theories of the cause of œdema. The theory commonly taught is that the kidney fails to excrete salt, and that, in consequence, water is retained to keep down the osmotic pressure of the body fluids; if this is what happens, the process of compensation must have been carried to excess in these cases, to produce the low electrolyte concentration found. The condition found appears more like a failure to excrete water with a compensatory retention of salt to keep up the concentration. This is more what might be expected on theoretical grounds, for it involves least work on the part of the kidney to excrete a urine containing salts in approximately the same concentration occurring in plasma; there should be no difficulty, therefore, in getting rid of some 10 grammes of sodium chloride a day, *if the power to excrete water is not impaired.*

The condition found in these cases does not resemble that found by Starling and Verney (1923) in their heart-lung-kidney preparations; for they found a free excretion of dilute urine, with a steadily increasing salt concentration in the plasma.

SUMMARY.

The concentration of electrolytes in the body fluids in nephritis with œdema is low, and may be definitely below the normal range. The bearing of this fact on theories of the cause of œdema is discussed.

I owe my thanks to the Physicians of the London Hospital for the facilities they have given to examine their patients.

REFERENCES.

CULLEN, G. E., AND ROBINSON, H. W.—(1924) *J. Biol. Chem.*, **61**, 832.
DENIS, W., AND HOBSON, S.—(1923) *Ibid.*, **55**, 183.
FISKE, C. H.—(1921) *Ibid.*, **48**, 63.
FODOR, A., AND FISCHER, C. H.—(1922) *Ztschr. f. d. ges. exper. Med.*, **29**, 465.
GRAM, H.—(1923) *J. Biol. Chem.*, **56**, 593.
LOEB, R., ATCHLEY, D., PALMER, W.—(1921) *J. Gen. Phys.*, **4**, 591.
MARRACK, J.—(1923) *Biochem. J.*, **17**, 240.
RABINOVITCH, I.—(1925) *J. Biol. Chem.*, **62**, 667.
SALVESEN, H., AND LINDER, G.—(1923) *Ibid.*, **58**, 617.
STARLING, E. H., AND VERNEY, H.—(1925) *Proc. R. Soc.*, B, **97**, 321.
VAN SLYKE, D. D.—(1922) *J. Biol. Chem.*, **53**, 495.—(1923) *Ibid.*, **58**, 523.
VAN SLYKE, D. D., WU, H., AND MACLEAN, H.—(1923) *Ibid.*, **56**, 765.